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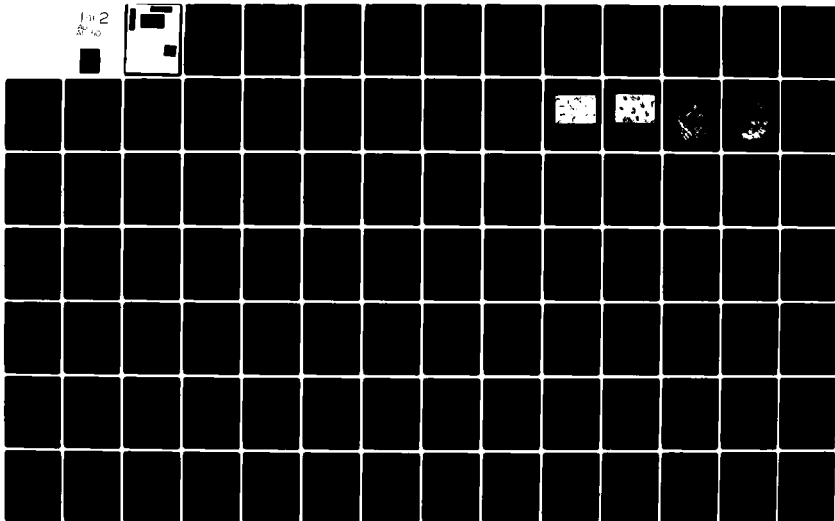
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Final Report
AFOSR-76-3069 Grant

Physiological and Anatomical Response
of Plant Leaf Tissue to Designated
Air Pollutants

O. Clifton Taylor
Principal Investigator

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A series of 22 experiments were conducted to determine the effects of hydrochloric acid (HCl) on the morphology, physiology, and metabolic processes of plants exposed to HCl mists. These studies were designed to elucidate the mechanisms which produce tissue damage. Histological, cytological and fine structural characteristics of plants exposed to HCl are described. Photosynthetic, respiratory and metabolic mechanisms for recorder from oxidant were also investigated. ←					

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Acknowledgments

I wish to express my thanks to Dr. Anton Endress for the excellent assistance in directing the research performed under this grant. As a Research Botanist, he had almost full responsibility for the research program until he accepted a position with the Illinois Institute of Natural Resources in 1979.

I wish also to thank Mrs. Sharon Suarez, John Kitasako and Theodore Swiecki for the excellent help as a technical staff. Special thanks is extended to Mrs. Suarez for her assistance in preparing this report.



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PHYSIOLOGICAL AND ANATOMICAL RESPONSE OF PLANT LEAF TISSUE TO DESIGNATED
AIR POLLUTANTS

I. Introduction

This is the fifth and final technical report of work performed under the sponsorship of the Air Force Office of Scientific Research Grant Number AFOSR-76-3069. These studies were initiated June 1, 1976 and designed to determine the mechanisms of gaseous HCl phytotoxicity. This report summarizes all the work that has been undertaken from June 1, 1976 to September 30, 1981. This project, entitled "Physiological and Anatomical Response of Plant Leaf Tissue to Designated Air Pollutants," was performed by members of the State-wide Air Pollution Research Center, University of California, Riverside, California 92521, Dr. O. Clifton Taylor, Principal Investigator.

II. PROGRAM OBJECTIVES

The long-range objective of the program was to determine precisely how atmospheric pollutants such as gaseous hydrogen chloride (HCl) produce damage to plant tissues. Five distinct facets of the program were:

1. To describe the histological, cytological, and fine structural characteristics exhibited by tissues exposed to pollutants at high, intermediate, and low concentrations.
2. To characterize physiological changes of fumigated tissues.
3. To correlate observed ultrastructural characteristics and measured physiological and metabolic activities by employing ultracytochemical techniques to observe fumigated tissues.
4. To examine structural and functional mechanisms for recovery from oxidant stress. Photosynthetic, respiratory, metabolic, and fine structural features were also studied.
5. To synthesize and evaluate the results to elucidate the mechanism(s) which produces tissue damage.

The broad approach of this program encompassed cytological, ultrastructural, biochemical, metabolic, physiological and organismic features.

For the 1980-1981 grant period, the following major research objectives were proposed:

1. Complete all pertinent studies of structural, biochemical, metabolic and physiological responses of plant tissues to gaseous HCl.
2. Integrate all research findings to assess the status of the program and to identify knowledge gaps or requirements for additional research observations.

3. Integrate experiment observations to formulate a mechanism for phytotoxicity.
4. Summarize our understanding of the impact of the space shuttle on vegetation.

III. STATUS OF RESEARCH EFFORT

The current status of our research activities, including projects initiated in previous grant periods, is summarized in Table 1. These studies include investigations into new aspects of plant responses to gaseous HCl, as well as a continuation of studies initiated earlier.

TABLE 1. STATUS OF AFOSR-76-3069 RESEARCH PROGRAM AS OF SEPTEMBER 30, 1980 (PAGE 1 of 15)

Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
STRUCTURAL STUDIES					
1. Light microscopic comparison of <u>Phaseolus vulgaris</u> primary leaves exposed for 20 min. to different concentrations of HCl gas					X Endress, Swiecki and Taylor, 1978
				X Swiecki, Endress and Taylor	
2. Light microscopic comparison of injury to <u>Phaseolus vulgaris</u> primary leaves treated with hydrochloric acid of differing pH					
3. Light microscopic comparison of injury to <u>Phaseolus vulgaris</u> primary leaves following exposure to acids at varying normal- ity (HCl, HNO ₃ , H ₂ SO ₄) to salts at varying molar- ity (NaCl, LiCl), or to polyethylene glycol.				X Swiecki, Endress and Taylor	
4. Ultrastructural appearance of <u>P. vulgaris</u> primary leaves one day after treatment for 20 min. with 25 mg HCl m ⁻³					X Endress, Kitasako and Taylor, 1978

TABLE 1 (continued - page 2 of 15)

Grant Year Research Initiated	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
					X Endress, Kitasako and Taylor, 1979
					X Endress, Kitasako and Taylor, 1979
					X Endress, Kitasako and Taylor, 1979 "a"
					X Endress, Kitasako and Taylor, 1979 "a"

5. Ultrastructural appearance of P. vulgaris primary leaves after treatment for 20 min.; concentrations of gaseous HCl ranging from 6.0 to 54.2 mg m⁻³. 1976

6. Ultrastructural analysis of injury development in P. vulgaris primary leaves following exposure to gaseous HCl (tissue sampled immediately, 1, 2, 4, 5, and 24 hrs. after HCl treatment, including reversible subcellular alterations. 1976

7. Ultrastructural detection of reversible and irreversible chloroplast alterations in P. vulgaris primary leaves following exposure to gaseous HCl 1976

8. Changes in cross-sectional area and length-width relationship of chloroplasts in P. vulgaris primary leaves exposed to HCl gas. 1976

TABLE 1 (continued - page 3 of 15)

	Grant Year Research Initiated	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
9. Effect of gaseous HCl treatments on chloroplast ultrastructure in leaves of <u>Tagetes erecta</u> (marigold).	1976					X Endress and Taylor, 1981
10. Ultrastructural detection of chloride ions in <u>P. vulgaris</u> primary leaf cells following treatment with differing concentrations of HCl gas.	1977					X Endress, Kitasako and Taylor, 1979b
11. Detection of moribund cells in leaves of <u>P. vulgaris</u> immediately after treatment with gaseous HCl using vital dyes	1977			X Experiment Discontinued		o
12. Role of leaf cuticle in determining susceptibility to HCl injury; effect of HCl on cuticle morphology and composition (<u>P. vulgaris</u> and <u>Citrus limon</u>)	1977					X Endress, Swiecki and Taylor
13. Ultrastructural detection of increased plasma membrane permeability using lanthanum markers in <u>P. vulgaris</u> (preliminary study)	1977			X Experiment Discontinued		

TABLE 1 (continued - page 4 of 15)

	Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
14. Subcellular localization of peroxidase activity in leaves of <u>P. vulgaris</u> and <u>L. esculentum</u> following treatments with ozone and gaseous HCl.	1978		X			
15. Ultrastructural analysis of injury development in <u>Spinacea oleracea</u> exposed to gaseous HCl	1978					X Endress and Taylor, 1981
16. Transmission electron microscopic analysis of cell wall structure and plasmodesmata in <u>P. vulgaris</u> leaves after HCl treatments	1979		X			
17. Scanning and transmission electron microscopic examination of trichome hydathodes on leaves of <u>P. vulgaris</u> .	1979		X			
18. Transmission electron microscopic analysis of the relationship between the ectodesmata in cell walls to the fragility of cell walls in HCl treated leaves	1980		X			

TABLE 1 (continued - page 5 of 15)

Grant Year Research Initiated	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
19.	Transmission electron microscopy study to localize sub-cellular site of cellulase enzyme activity in HCl-treated leaves		X		
20.	Transmission electron microscopy study to examine cellulase activity with respect to changes in cell wall ultrastructure		X		
METABOLIC STUDIES					
1.	Accumulation of chloride ions in leaves of <u>P. vulgaris</u> following single, 20 min. exposures to HCl gas of variable concentration.				X Endress, Kitasako and Taylor, 1979b
2.	Accumulation of chloride ions in different aged leaves of <u>P. vulgaris</u> following single, 20 min. exposure to HCl gas of variable concentration.				X Endress, Kitasako and Taylor, 1979b

TABLE 1 (continued - page 6 of 15)

Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
1976	3. Relationship between level of accumulated chloride and visible injury to <u>P. vulgaris</u> primary leaves.				X Endress, Kitasako and Taylor, 1979b
1976	4. Relationship of HCl dose and level of accumulated chloride in leaves of <u>P. vulgaris</u> and <u>Raphanus sativa</u> (radish).	X Experiment Discontinued.			
1976	5. Relationship between quantities of accumulated chloride in primary leaves of <u>P. vulgaris</u> following treatment with gaseous HCl administered continuously or intermittently	X Experiment Discontinued			
1976	6. Changes in chlorophyll content of <u>P. vulgaris</u> primary leaves following 20 min. HCl treatments.			Endress, Suarez and Taylor	
1977	7. Changes in protein content of <u>P. vulgaris</u> primary leaves following 20 min. HCl treatments			Endress, Suarez and Taylor	

TABLE 1 (continued - page 7 of 15)

Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
1977			X Experiment Discontinued		
1980		X			
1977					
1977					
1978					
PHYSIOLOGICAL STUDIES					
1977					
1977					
1978					

8. Changes in nucleic acid levels of P. vulgaris primary leaves following 20 min. HCl treatments.

9. Studies on protein levels in leaves following HCl treatments by examining free amino acid pools.

10. Determination of proline levels in plants stressed by withholding water and/or exposure to HCl gas

PHYSIOLOGICAL STUDIES

1. Photosynthetic activity of chloroplasts isolated from P. vulgaris primary leaves exposed to HCl gas.

2. Photosynthetic activity of leaf discs taken from P. vulgaris primary leaves exposed to HCl gas.

3. Photosynthetic activity of chloroplasts isolated from S. oleracea leaves exposed to HCl gas.

TABLE 1 (continued - page 8 of 15)

Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
4. Photosynthetic activity of leaf discs taken from <u>S.</u> <u>oleracea</u> leaves exposed to HCl gas	1978			Endress, Suarez and Taylor	
5. Oxygen evolving capacity of isolated chloro- plasts at different pH.	1978			Endress, Suarez and Taylor	
6. Incidence of foliar injury to <u>P. vulgaris</u> primary leaves following exposure to HCl gas in the dark (pre- liminary study).	1977	X Experiment Discontinued			11
7. Does abscisic acid (causes closure of stomates) treat- ment reduce injury on <u>P.</u> <u>vulgaris</u> primary leaves ex- posed to gaseous HCl?	1977	X Experiment Discontinued			
8. Altered membrane permeabil- ity in discs from <u>P. vul-</u> <u>garis</u> primary leaves exposed to gaseous HCl.	1977				X Heath and Endress, 1979
9. Does dessication stress to <u>P. vulgaris</u> plants in- fluence susceptibility of leaves to HCl injury or injury severity?	1978	X			

TABLE 1 (continued - page 9 of 15)

	Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
10. Does desiccation stress to <u>Raphanus sativus</u> (radish) influence susceptibility of leaves to HCl injury or injury severity?	1978			X Experiment Discontinued		
11. Determination of the water saturation deficit in <u>P.</u> <u>vulgaris</u> primary leaves not exposed to air pollutants.	1978			X		
12. Water status and stress studies to assess the role of stomates and water deficits in mediating HCl injury	1980		X			
13. Photorespiration activity of leaf discs taken from <u>P. vulgaris</u> primary leaves exposed to HCl gas	1980			X		
14. Determination of the cellular pH of leaf cells exposed to gaseous HCl.	1980			X Experiment Discontinued		

TABLE 1 (continued - page 10 of 15)

Grant Year Research Initiated	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
BIOCHEMICAL STUDIES					
1. Relationship between visible foliar injury on primary leaves of <u>P. vulgaris</u> plants and activity of peroxidase enzyme following exposure					X Endress, Suarez and Taylor, 1979
2. Relationship between visible foliar injury on primary leaves of <u>P. vulgaris</u> plants and activity of peroxidase enzyme following exposure to ozone.	1978				X Endress, Suarez and Taylor, 1979
3. Relationship between visible foliar injury on leaves of <u>Lycopersicon esculentum</u> (tomato) and activity of peroxidase enzyme follow- ing exposure to gaseous HCl.	1978				X Endress, Suarez and Taylor, 1979
4. Relationship between visible foliar injury on leaves of <u>L. esculentum</u> and activity of peroxidase enzyme follow- ing exposure to ozone.	1978				X Endress, Suarez and Taylor, 1979
5. Analysis of isozyme patterns of peroxidase enzyme from <u>P. vulgaris</u> and <u>L. esculentum</u> following treatments with ozone and gaseous HCl.	1978				X Endress, Suarez and Taylor, 1979

TABLE 1 (continued - page 12 of 15)

Grant Year Research Initiated ^a	Research in Progress	Research Completed, but Data not in Manuscript Draft	Data Incorporated in a Manuscript Draft	Data Contained in a Submitted Manuscript	Data Contained in a Publication or Manuscript Accepted for Publication
OTHER STUDIES					
1. Extent of visible foliar injury on primary leaves of 8-day old <u>P. vulgaris</u> plants as function of gaseous HCl concentration.	1976				X Endress, Swiecki and Taylor, 1978
2. Distribution of abaxial glazing and interveinal necrosis on 8-day old <u>P. vulgaris</u> dependent on primary leaves related HCl concentration	1976				X Endress, Swiecki and Taylor, 1978
3. Age-dependent susceptibility of <u>P. vulgaris</u> primary leaves to injury from HCl gas	1976				X Endress, Oshima and Taylor, 1979
4. Effect of a single 20 min. exposure to HCl gas on subsequent expansion of primary leaves of <u>P. vulgaris</u> .	1976				X Endress, Oshima and Taylor, 1979
5. Effect of repeated HCl exposures on growth and yield of sweet peas (<u>Lathyrus odoratus</u>).	1977		X Experiment Discontinued		

^a 1976 Grant Period was from June 1, 1976 to May 31, 1977; 1977 Grant period was from June 1, 1977 to May 31, 1978; 1978 Grant Period was from June 1, 1978 to May 31, 1979; 1979 Grant Period was from June 1, 1979 to May 31, 1980.

A. Morphological Studies

1. Scanning Electron microscopic examination of trichome hydathodes exposed to HCl gas.

Trichome hydathodes dot the surface of pinto bean leaves. These are small structures, consisting of a circular "head" of about four cells supported above the leaf surface by a single stalk cell. Trichome hydathodes were described nearly a century ago, but very few investigations have been concerned with them in modern times. Although they bear no resemblance to hydathodes, they were named accordingly to their hypothesized role as specialized sites for water loss. Indeed our observations from scanning electron microscopy indicated that the trichome hydathodes on the bean leaf surface appear to be quite sensitive to gaseous HCl as they are generally the first structures to be injured. See Figures 1 and 2.

2. Subcellular localization of peroxidase and catalase activity in leaves of *P. vulgaris* fumigated with gaseous HCl.

The localization of peroxidase and catalase in pinto bean leaves exposed to HCl gas was repeated (See 1980 Annual Report for description of experiment) to determine whether differences in enzyme levels between control and treated plants could be discerned. Examples of treated and control samples are presented in Figure 3 and 4. It appears that differences in the enzyme levels can be detected although quantification of the enzyme levels is difficult.

Figure 1. Scanning Electron micrograph of trichome hydathodes, control.

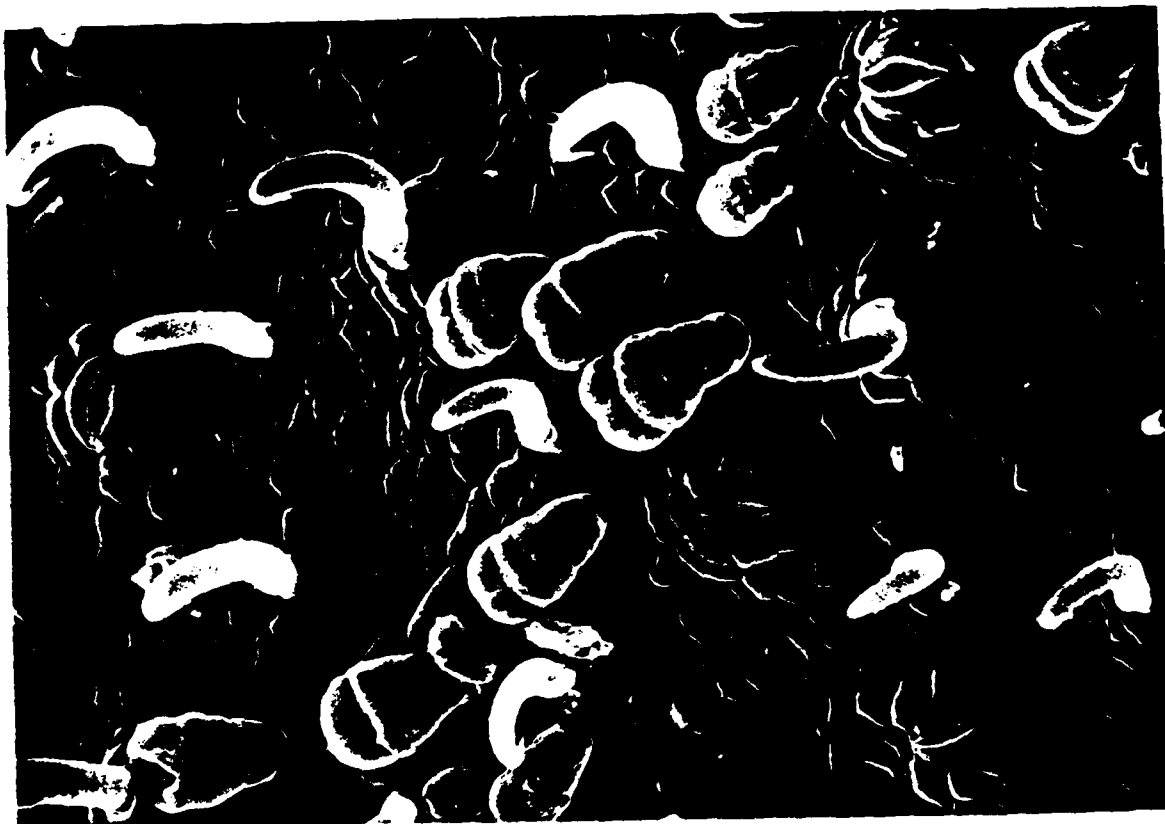
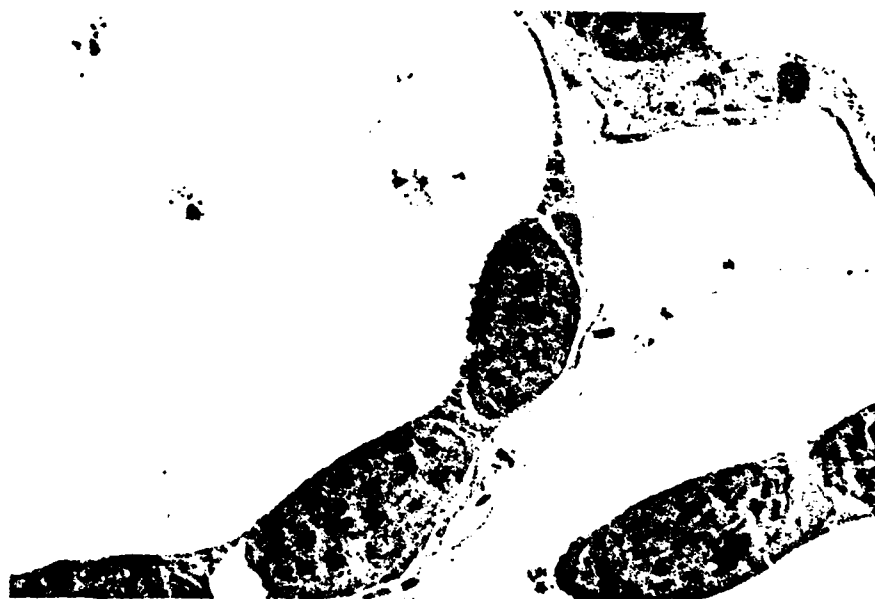


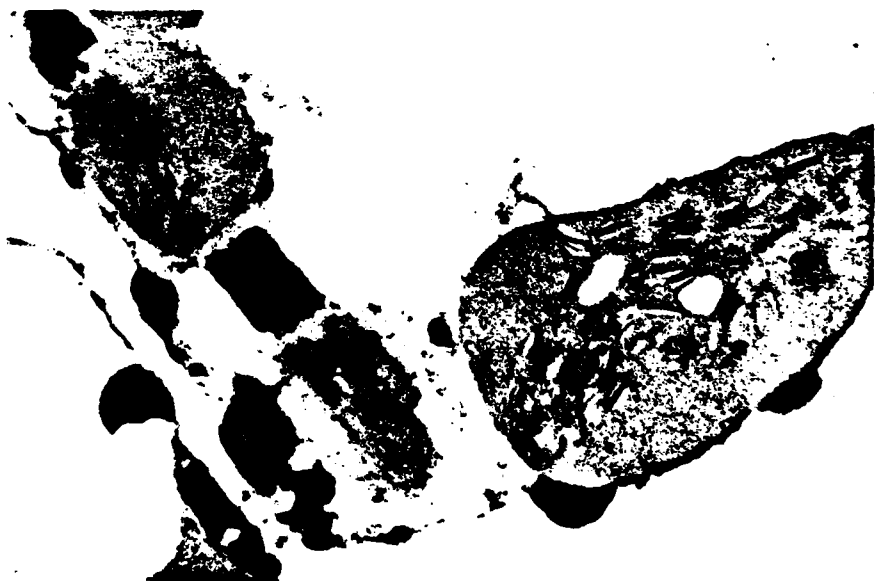
Figure 2. Scanning Electron micrograph of trichome hydathodes, HCl exposed.



Figure 3. Subcellular location of peroxidase activity in leaves of Phaseolus vulgaris, cv. Pinto exposed to HCl gas. Areas of electron dense material in tissue treated with HCl represent sites of increased activity.

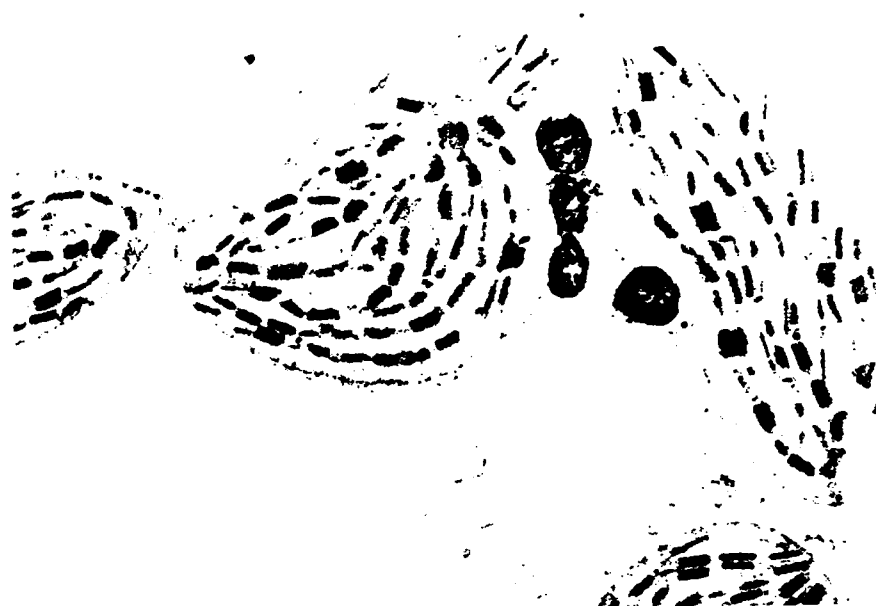


Control



HCl treated

Figure 4. Subcellular localization of catalase activity in leaves of Phaseolus vulgaris cv. Pinto grown in carbon-filtered air and exposed to HCl gas. Electron dense areas represent sites of catalase activity. HCl treated cell contents are disrupted and have shrunk away from the cell wall.



Control



HCl treated

B. Metabolic Studies

1. The effect of HCl gas on cellulase activity

Because of cellulase's role in the senescence of plants and leaf drop, its response to HCl exposure was examined. Either control and HCl exposed leaves or abscission zones were harvested, weighed and homogenized, using a Waring blender, in 0.02 M Tris pH 8.1, 3mM EDTA and 1 M NaCl. Samples were then filtered through cheesecloth, centrifuged at 13K for 20 and analyzed for cellulase activity. Viscometric measurement of cellulase activity units was performed according to the method of

Experimental results are reported in Table I. In experiment 1, the total cellulase activity in pinto bean leaves was measured at 0 hours post fumigation and, in the abscission zones at 6 days post fumigation. Cellulase activity in leaves exposed to both high and low concentrations of HCl gas was significantly (5% level) lower than in control leaves. No significant differences between control and treated plants were found in the abscission zones 6 days after HCl exposure, although there was a slight increase in treated plants.

In experiment 2, experimental procedures used to induce cellulase in the abscission zone with ethylene were followed to determine if HCl gas acted in a similar manner. The leaves of 12 day old pinto beans were removed just above the abscission zones; the stems were exposed to HCl gas and cellulase activity in the abscission zones was determined 4 days later. No significant difference between control and treated plants was observed.

Cellulase levels from control plants are in line with what is predicted; the enzyme activity is normally high in the abscission zones of plants and

TABLE I. Cellulase activity in P. vulgaris exposed to gaseous HCl.Experiment 1:

	<u>Control</u>	<u>HCl-Exposed</u>	
HCl concentration (mg M ⁻³)	0	18	23.4
Total units cellulase per mg protein per leaves at 0 hours post- fumigation	0.225 ± .129	0.112 ± .045	0.579 ± .028
Significance		5%	5%
Average damage Rank of Beans		3.7	4.2

Total units cellulase per mg protein per abscission zones of bean leaves (6 days post fumigation)	11.28	12.18	13.82

Experiment 2:

HCl concentration (mg M ⁻³)	0	24.9
Total units of cellulase per mg protein per fumigated abscission zones (4 days post fumigation)	107.1	94.55
Significance		N.S.

low in the leaves. Higher than normal levels can be attained in the abscission zones if the plant's leaves are removed prior to the analysis. Experimental results suggest that HCl gas has no direct effect on cellulase activity. The drop in activity in the leaf may be due either to the acidification of the external leaf area or a loss of water from the leaf, both of which may have the tendency to slow down or destroy enzyme production or activity. In the more critical area of the plant, the abscission zone, which is responsible for leaf drop, cellulase activity was unchanged. This area is a much smaller and better protected area of the plant (thicker walls) and thus is less likely to suffer damage from HCl gas.

2. The effect of HCl gas on free amino acid levels

Eight- and 12-day old pinto beans were fumigated with HCl gas for either 20 minutes at 0, 15, 25 or 40 mg m^{-3} HCl or 1, 2 or 4 hours at either 0 or 15 mg m^{-3} HCl. At both 0 and 24 hours post fumigation, one leaf per plant was removed, pooled into treatments (10 leaves total per treatment) and frozen in liquid nitrogen. Samples were lyophilized and then crushed into a fine powder. Free amino acid extracts were prepared according to the methods of Bligh and Dyer (1959). 200 mg of sample was mixed with 7 ml of water for 30 minutes; 26 ml of chloroform/methanol (1:2) was added and mixed for another 30 minutes; 8.4 ml of water was added and the solution mixed 5 minutes. Finally, 5 ml of chloroform was added, stirred for 5 minutes, then centrifuged at 3000 RPM for 10 minutes. The supernatant was layered onto a Dowex 50 w resin bed and washed with water. Amino acids were eluted with cold NH_4OH followed by a water rinse. Samples were lyophilized, then brought up in water and analyzed for free amino acids using an amino acid analyzer. Results of the changes in amino acids observed are presented in Table 2 and Figure 5.

TABLE 2. Changes in Free Amino Acids from Control Levels at 0 hrs
Post Fumigation

AMINO ACIDS	12 Day Old Pinto Beans						8 Day Old Pito Beans					
	HCl mg M ⁻³ , 20 min.			15 mg m ⁻³ HCl per X hours			HCl, mg M ⁻³ , 20 min			15 mg m ⁻³ HCl per X hours		
	15	25	40	1	2	4	15	25	40	1	2	4
Lysine	0	+	+	0	0	+	-	-	+	+	+	+
Histidine	-	+	+	-	+	+	+	+	-	+	-	+
Ammonia	+	-	-	-	-	+	+	+	+	+	+	+
Aspartic Acid	-	-	-	-	-	-	-	-	-	-	-	-
Threonine	-	+	+	0	+	+	+	+	+	+	0	+
Serine	-	+	-	+	+	+	+	+	-	+	-	+
Glutamic Acid	-	-	-	-	-	-	0	+	-	0	-	-
Proline	0	+	+	+	+	+	-	-	-	+	+	+
Glycine	-	-	-	+	+	-	+	+	-	+	-	+
Alanine	0	+	+	+	-	+	+	+	-	+	-	+
Valine	+	+	+	+	+	+	+	+	-	0	0	+
Isoleucine	-	+	+	0	+	+	+	+	-	+	+	+
Ileucine	+	+	+	+	+	+	+	+	-	+	+	+
Tyrosine	0	+	+	0	+	+	0	0	0	0	+	+
Phenylalanine	-	+	-	+	0	+	+	+	-	+	0	+

Key: + = Increase

- = Decrease

0 = No change

Figure 5. Free amino acid levels in pinto beans exposed to HCl.

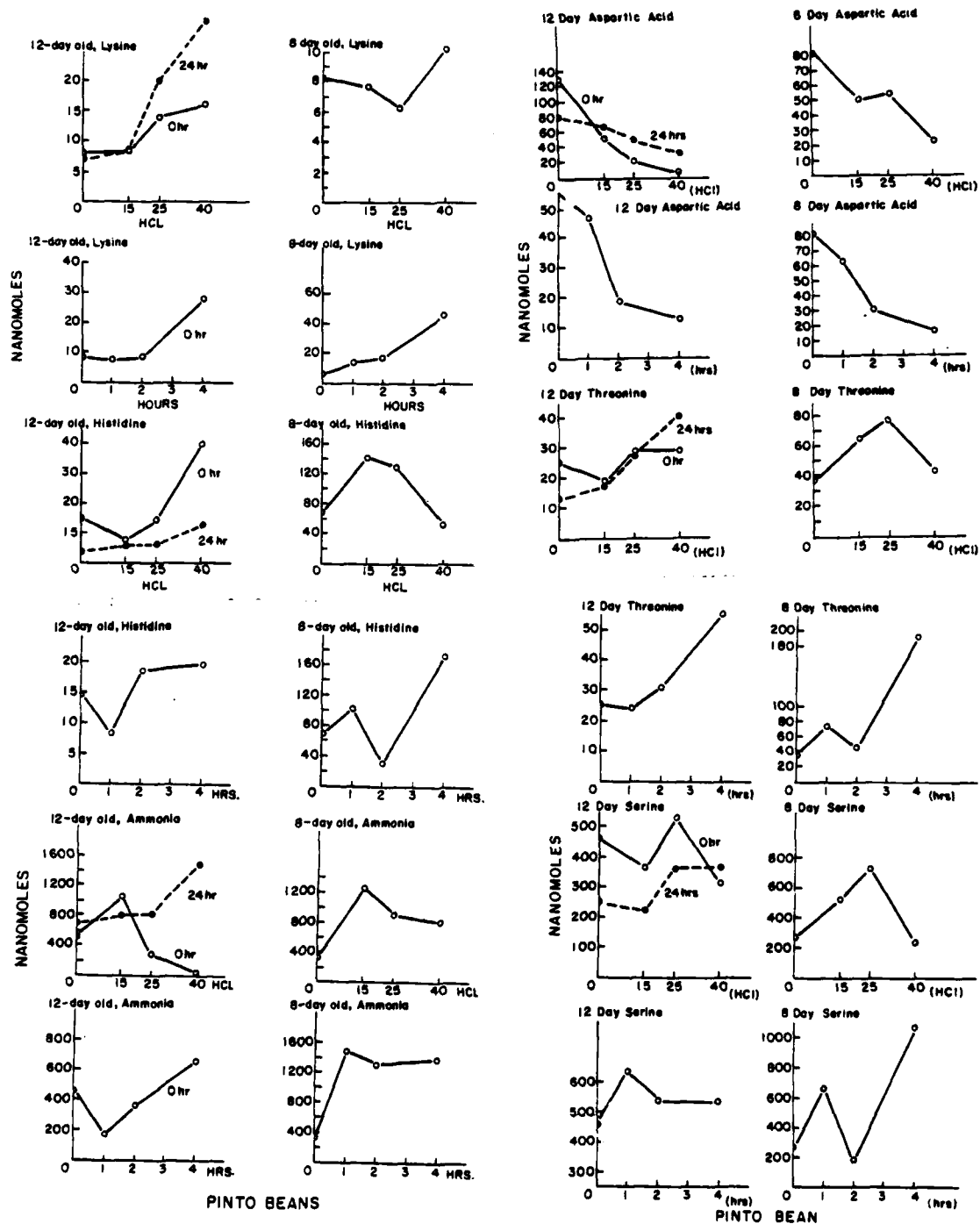


Figure 5 Continued (Page 2 of 3)

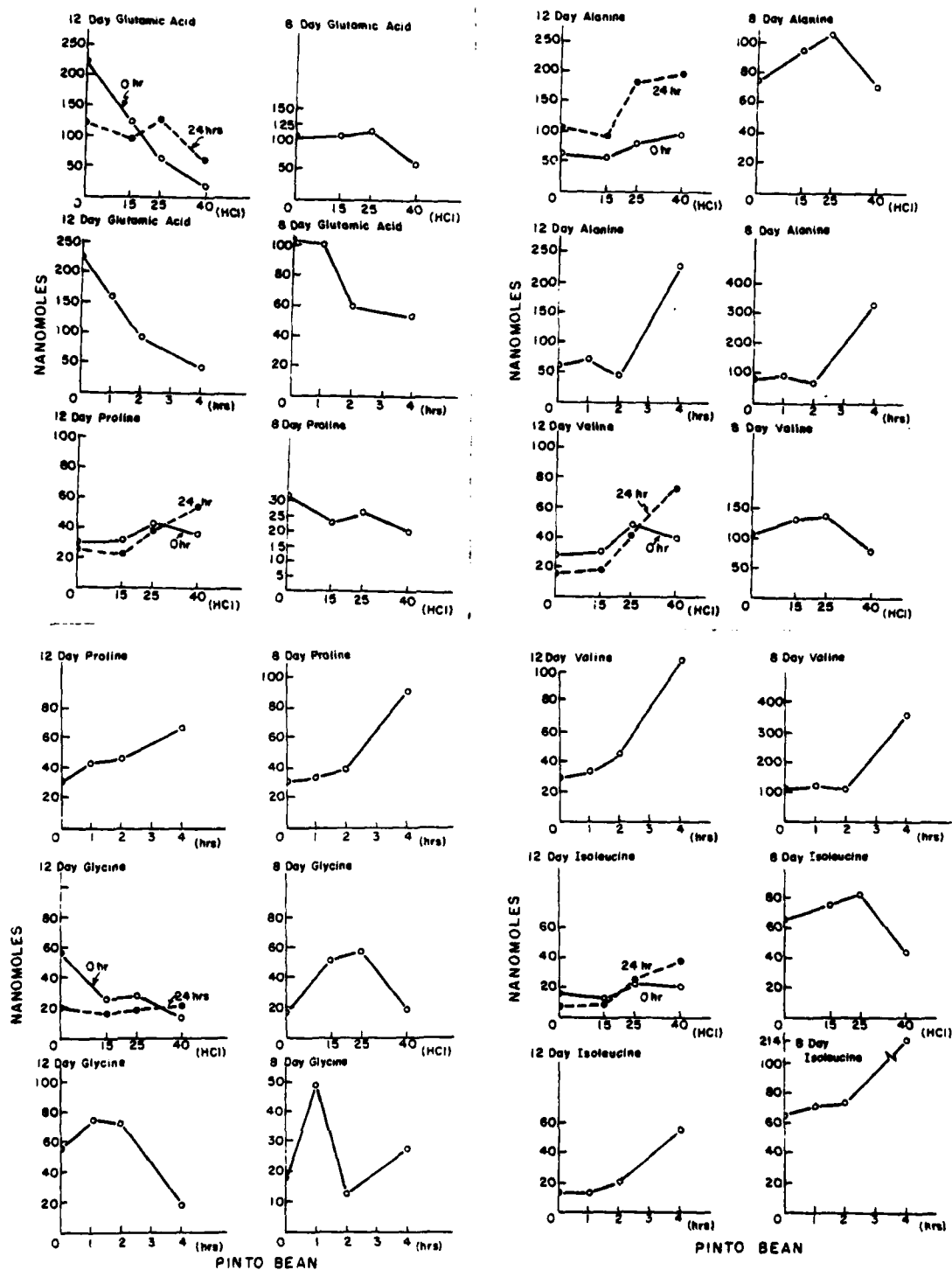
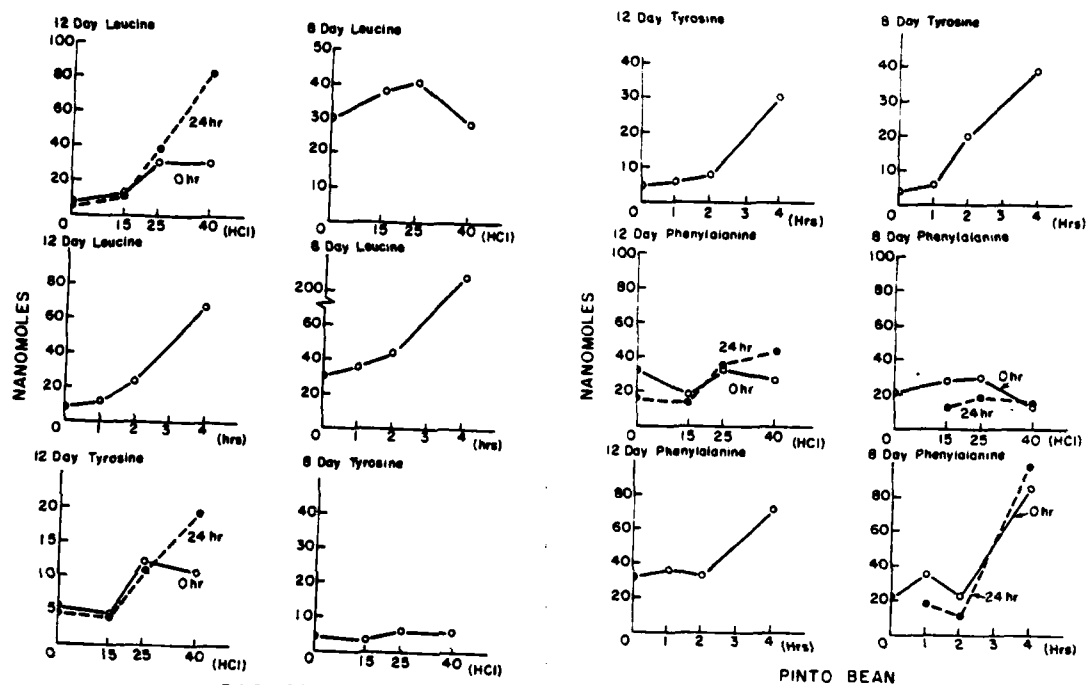


Figure 5 Continued (Page 3 of 3)



C. Physiological Studies

1. The effect of HCl gas on Photorespiration and related metabolic activities

The results of these studies are presented in the following incomplete manuscript draft. This is a first draft and lacks both the introduction and discussion sections. The results from the photorespiration studies support both our previous hypothesis that HCl exposure results in an acidification of the cytoplasm and our results from previous studies on photosynthesis and respiration (Annual Report, 1980). HCl gas was shown to cause a decrease in photosynthetic and respiratory activity in plants sustaining between 20 and 85% necrotic injury; and, a decrease in the photosynthetic activity of isolated chloroplasts was observed as their incubating media was made more acidic. (Endress et al., 1980). Correspondingly, if the apparatus is present, an increase in photorespiration is expected as photosynthesis is decreased (Zelitch, 1968). This is in fact what is observed.

Glycolic acid oxidase, a key enzyme in photorespiration, was also measured. The enzymatic activity observed corroborated our photorespiration studies. Glycolic acid oxidase activity was significantly higher in plants that exhibited more than 40% injury.

PHOTORESPIRATION IN PHASEOLUS VULGARUS
EXPOSED TO GASEOUS HYDROGEN CHLORIDE

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Received , 1980

Running Title: HCl Effects on Photorespiration

PHOTORESPIRATION

Materials and Methods:

Plant material. Pinto bean (*Phaseolus vulgaris* L. var. V.I. III) plants were grown from seed in a greenhouse cooled by evaporative coolers. Air entering the greenhouse was filtered through activated charcoal. Plants were established in 10 cm pots containing a soil medium of equal parts sandy loam, peat and redwood shavings. Plants were fertilized weekly with 120 ml/pot micronutrient solution (Hoagland and Amon, 1950). During the course of these studies, daily temperature maxima fluctuated within the range of 24-38°C during daylight hours and were held above 20°C at night. Both 8 and 12 day old pinto beans were used in the photorespiration studies and 12 day old pinto beans were used in the enzyme assays.

Fumigation conditions. Exposures to anhydrous HCl were in 2.03 m³ cylindrical continuous stirred tank reactors (CSTR)³ (located within the greenhouse) modeled after those of Rogers et al. (1977). Each chamber received 1.5 changes of air per minute. Anhydrous HCl was obtained commercially and metered into the CSTR through rotameters. To measure HCl concentration, a 15 liter volume of reactor chamber air was bubbled through 0.1N HNO₃ and the chloride content of this solution was determined with an Aminco automatic chloride titrator. Control plants were placed simultaneously in identical tank reactors devoid of HCl. Treated plants used in both the leaf disk photorespiration assay and enzyme assays were exposed to 20 mg m⁻³ HCl for 30 minutes. At the end of the exposure period, plants were either sampled immediately or returned to greenhouse benches to be used in estimating the visible injury. Visible foliar injury was measured 24 hours after treatment by scoring the leaves for the

presence or absence of necrosis and/or abaxial glazing, estimating the proportion of necrotic leaf surface area, and accordingly assigning an arbitrary injury rank: 0 (0 necrotic leaf surface), 1 (0 X 20%), 2 (20% X 40%), 3 (40% X 60%), 4 (60% X 80%), or 5 (80% X 100%).

Photorespiration assay. Photorespiration was measured according to the method of Zelitch (1968). Six leaf disks (number 4 punch) per sample were floated on 1 ml of deionized water in a 15 ml, single arm warburg vessel. 50 μ l of 1000 μ M $\text{NaH}^{14}\text{CO}_3$ (specific activity 48 $\mu\text{Ci}/\mu\text{mol}$) was added to the side arm which was then sealed with a serum stopper. One flask, prepared as outlined above minus the leaf disks, was routinely included with each experiment. All the warburg vessels were placed in a constant temperature water bath at 30°C and shaken for 45 minutes in the light. The vessels were open to the air during this time. Zelitch (1968) observed that during this period, stomata would open and that subsequent uptake of $^{14}\text{CO}_2$ would occur at a rapid rate. The vessels were then closed to the atmosphere and $^{14}\text{CO}_2$ was liberated by injecting acid through the rubber stopper into the $\text{NaH}^{14}\text{CO}_2$ solution. Zelitch (1965) demonstrated that leaf disks can assimilate all the $^{14}\text{CO}_2$ in approximately 15 minutes. A total of 30 minutes under this closed system was allowed to elapse before photorespiration measurements were begun.

With a bank of lights on, photorespiration was measured by passing CO_2 -free air (air passed through 250 ml of 2.5 N KOH and water successively) through the warburg flasks into 15 ml of 1.0 M ethanolamine, which traps $^{14}\text{CO}_2$ that has been released, for 35 minutes. The lights were then turned off and a sample of $^{14}\text{CO}_2$ released in the dark was collected. An aliquot of each sample was added to 2 ml of aquasol and counted using a liquid scintillation counter. A ratio of $^{14}\text{CO}_2$ released in the light to that released in the dark was calculated.

Enzyme assay. One leaf per plant from 12 day old pinto beans was removed prior to HCl exposure and its corresponding leaf was removed either 0 or 24 hours post treatment. Each leaf was homogenized in 8 ml of cold, extraction buffer (0.04M Tris pH 7.8, 0.01 M MgCl_2 and 0.25mM Edta and 5 mM GSH). The homogenate was centrifuged at 20,000 x g for 10 minutes and the resulting supernatant was analyzed for enzyme activity. A 1 ml aliquot of each sample was analyzed for protein according to the method of Lowry et al. (1951).

Glycolate oxidase enzyme activity was determined according to the method of Crookston et al. (1974). 50 μl of enzyme extract, 775 μl of 0.1 M KH_2PO_4 , pH 7.4 and 75 μl of 0.05 M phenylhydrazine HCl were combined in a spectrophotometer, quartz cuvette; 50 μl of 0.1 M glycolate was added and the production of phenylhydrazone was measured at 324 nm.

Malate dehydrogenase enzyme activity was measured according to the methods of Hedley and Stoddart (1971). 15 μl of enzyme extract, 200 μl of 1mM oxaloacetate and 1000 μl of 0.1 M KH_2PO_4 , pH 7.5 were combined in a spectrophotometer, quartz cuvette; 10 μl of 0.03 M NADH was added and the loss of absorption at 340 nm due to the oxidation of NADH was recorded.

Results:

Results of the leaf disk assays measuring photorespiration are as follows:

1. The mean photorespiration rate of 12 day old pinto beans fumigated with HCl was significantly increased (5%) (Table 1).
2. A graphical analysis of the data indicates that the increase in photorespiration occurs in plants exhibiting 20% or more injury (Figure 1). Photorespiration levels in plants exhibiting less injury was either near or slightly below control levels.

3. There was no significant difference in the photorespiration rates of 8 and 12 day old, untreated pinto beans.
4. There was no significant difference in photorespiration rates between control and HCl treated 8 day old pinto beans exhibiting less than 20% damage.

TABLE 1. Photorespiration of leaf disks from both control and treated 8 and 12 day old Pinto beans. Treated plants were exposed to 20.56 mg m⁻³ HCl for 30 min.

Sample expt. no	Injury	¹⁴ CO ₂ released in light: Control	¹⁴ CO ₂ released in dark Treated	% Control
<u>12 day old pinto beans</u>				
1	.125	1.54	.835	54.4
2	.25	4.76	5.14	108
3	1.82	.61	.66	109
4	2.25	1.11	2.36	213
5	2.28	1.29	16.8	1300
6	2.5	1.8	16.6	922
7	2.7	1.07	3.68	344
8	2.94	1.94	1.77	91
9	3.69	1.06	12.82	1210
10	4.9	.53	2.6	490
11	5.0	1.54	12.06	783
Mean		1.56 ± 1.09	6.85 ± 3.7 *	
<u>8 day old pinto beans</u>				
1	.5	1.43	.89	62.5
2	.5	1.15	1.65	144
3	.82	1.79	1.43	80
4	4.62	.78	28	3619
Mean		1.29 ± .43	7.99 ± 13.3	

*significant level of difference of 5% as determined by the students' t-test.

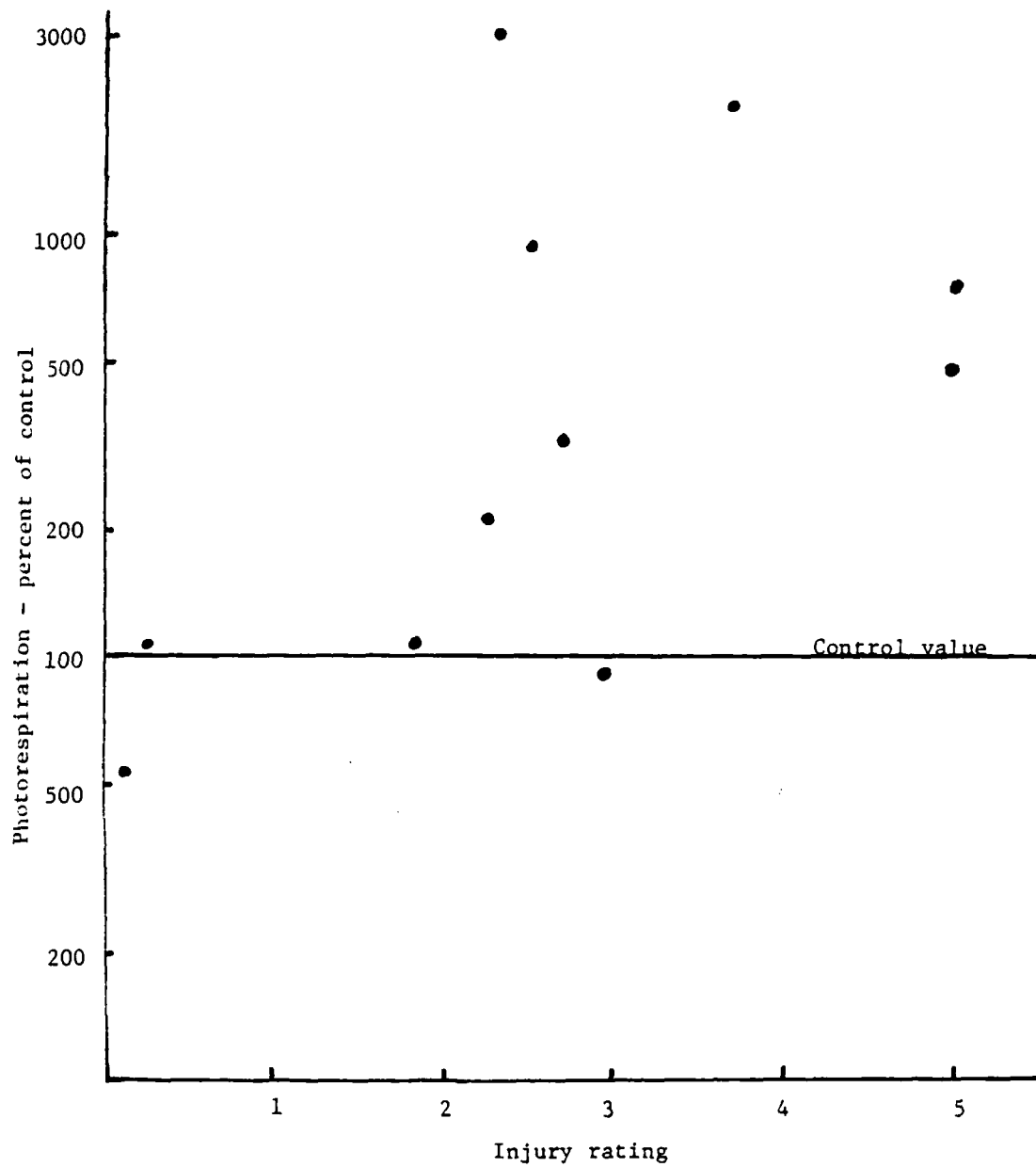


Figure 1. Amount of photorespiration by 12 day old pinto bean plants exposed to HCl gas compared to control plants. Increased photorespiration occurred when the leaf injury rating was greater than 20 %.

Results of enzyme assays:

In an attempt to minimize plant-to-plant variation, paired samples were used for enzyme analysis. Each extract of each leaf sample was analyzed for both glycolic acid oxidase activity and malate dehydrogenase activity (Table 2).

Glycolic acid oxidase was significantly (5%) increased both 0 and 24 hours post fumigation, sampling when plants exhibited more than 40% leaf injury.

In plants with less than 20% injury, experiment 2, both the control and HCl exposed plants were significantly (5%) higher in glycolic acid oxidase activity than their paired bench samples at 0 hours post fumigation. There was no difference between the air and treated samples. This suggests that something other than HCl, perhaps some environmental factor, was responsible for this increase.

Malate dehydrogenase activity increased significantly (5%) in the 24 hours post fumigated plants that exhibited greater than 40% damage and decreased significantly (5%) in the 24 hours post fumigated plants that exhibited less than 20% injury (Table 2).

TABLE 2. Effect of HCl exposure on the activity of two enzymes - glycolic acid oxidase and malate dehydrogenase.

GLYCOLIC ACID OXIDASE					MALATE DEHYDROGENASE				
EXPERIMENT	$\Delta OD/\text{MIN}/\text{MG PROTEIN} + \text{STANDARD ERROR}$				$\Delta OD/\text{MIN}/\text{MG PROTEIN} + \text{STANDARD ERROR}$				DAMAGE RANK
	BENCH	TREATMENT	0h POST-TREATMENT	24 hr POST-TREATMENT	BENCH	TREATMENT	0hr POST-TREATMENT	24 hr POST-TREATMENT	
I	166.3 \pm 45	AIR	189.7 \pm 38		2.49 \pm .22	AIR	2.94 \pm .24		
	165.1 \pm 26	AIR		191.3 \pm 54	2.62 \pm .15	AIR		3.12 \pm .63	
	148 \pm 31	HCl	192.8 \pm 36*		2.39 \pm .19	HCl	2.83 \pm .22		
	144 \pm 25	HCl		210 \pm 43*	2.43 \pm .19	HCl		3.21 \pm .22*	>2
II	61.6 \pm 5.6	AIR	84.6 \pm 21*		.515 \pm .04	AIR	.542 \pm .13		
	65.9 \pm 17.4	AIR		85.8 \pm 19	.500 \pm .18	AIR		.227 \pm .06	
	54.3 \pm 20.4	HCl	71.2 \pm 20.2*		.457 \pm .21	HCl	.559 \pm .085		
	62.6 \pm 19.9	HCl		82.4 \pm 26	.593 \pm .16	HCl		.258 \pm .12*	<1

* 5% significant difference between treated and corresponding bench samples.

Plants were exposed to 20.93 mg m⁻³ HCl for 30 minutes.

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2. The effect of HCl gas on free proline levels and water dessication

In the 1977-1978 grant period studies examining the effects of HCl gas on the water status of Phaseolus vulgaris were begun and preliminary results were reported in the 1980 annual report. These observations have been combined with studies examining the relationship between HCl exposure and free proline levels and, are presented in the manuscript that follows.

The Relationship Among Gaseous HCl Exposure, Water Deficits and
Proline Levels in Phaseolus vulgaris

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Introduction

Increases in free proline levels in response to water stress, salinity, and low temperatures have been reported for a number of plant species, including several important agricultural crops. Upon removal of the stress, levels drop rapidly (Singh et al., 1972; Singh et al., 1973; Chu et al., 1974; Chu et al., 1976; Palfe and Juhasz, 1971; Stewart and Lee, 1974; Barnett and Naylor, 1966; Kemble and MacPherson, 1954; Routley, 1966; Stewart et al., 1966). Proline levels, which are usually low and tightly regulated in grasses, appear to present a universal response by increasing when stress is applied (Hanson, 1980). Because proline is highly labile, considerable effort has been directed towards incorporating this response in methods that could be used to measure crop water stress and ultimately determine crop irrigation schedules and to breed cereals for drought resistance (Palfe and Juhasz, 1971; Singh et al., 1972). Possible explanations for the accumulation of proline that have been studied include: a stimulation of proline synthesis from glutamic acid due to the loss of the feedback inhibition of the synthesis of intermediate Δ^1 -pyrroline-5-carboxylate (P-5-C), inhibition of proline oxidation, impairment of the incorporation of free proline into protein, and a reduction in the export of proline via the Phloem (Hanson, 1980).

This paper examines the relationship between gaseous HCl exposure to plants and the resulting effects that it has on both the plant's water status and proline levels to determine whether proline can be used as a metabolic indicator in assessing HCl damage.

Stress

Materials and methods

Plant material. Pinto bean (*Phaseolus vulgaris* L. var vI III) plants were grown from seed in a greenhouse cooled by evaporative coolers. Air entering the greenhouse was filtered through activated charcoal. Plants were established in 10 cm pots containing a soil medium of equal parts sandy loam, peat and redwood shavings. Plants were fertilized weekly with 120 ml/pot micro-nutrient solution (Hoagland, 1950). During the course of these studies, daily temperatures fluctuated within the range of 24-38°C during daylight hours and were held above 20°C at night. Both 8 and 12 day old pinto beans were used in these studies.

Fumigation conditions. Exposures to anhydrous HCl were in 2.03 m³ cylindrical continuous stirred tank reactors (CSTR)³ (located within the greenhouse). Each chamber received 1.5 changes of air per minute. Anhydrous HCl was obtained commercially and metered into the CSTR through rotameters. To measure HCl concentration, a 15-liter volume of reactor chamber air was bubbled through 0.1 N HNO₃ during the 20 to 30-minute fumigation period, and the chloride content of this solution was determined with an Aminco automatic chloride titrator. Treated plants were exposed to varying concentrations of HCl ranging from 15 to 45 mg m⁻³ for either 20 or 30 minutes depending upon the experiment. Control plants were placed simultaneously in identical tank reactors devoid of HCl. At the end of the exposure period, plants were either sampled immediately or returned to greenhouse benches to be used subsequently or to be used in estimating visible injury. Visible foliar injury was measured 24 hours after treatment by scoring the leaves for the presence or absence of necrosis and/or abaxial glazing, estimating the proportion of necrotic leaf surface area, and assigning an arbitrary injury rank: 0 (no necrosis), 1 (0<X<40%), 3 (40%<X<60%),

4 ($60\% < X < 80\%$), and 5 ($80\% < X < 100\%$).

Free proline assay. Free proline was determined according to the method of Bates, Waldren and Teare (1973). One leaf per plant (approximately 0.5-1.0 gm) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at 10,000 rpm for 10 minutes. Two ml of the supernatant was reacted with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 hour at 100°C. Samples were placed in an ice bath to terminate the reaction. The reaction mixture was extracted with 4 ml of toluene, mixing vigorously using a vortex for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and its absorbance read at 520 nm using toluene as a blank.

The acid-ninhydrin was prepared by warming 1.25 gm ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid. This solution will remain stable for 24 hours when stored at 4°C (Troll and Lindsley, 1955). However, for these experiments, the solution was made fresh each time that it was used.

Proline concentration was determined from a standard curve prepared at the same time the samples were analyzed. Calculations on a fresh weight basis were as follows:

$$[(\mu\text{g proline/ml} \times \text{ml toluene}) / 115.5 \mu\text{g}/\mu\text{mole}] / [(\text{gm sample})/5] = \mu\text{moles proline/gm of fresh weight material.}$$

Determination of water status. Water deficit was determined by measuring relative turgidity according to the method of Barrs and Weatherly (1962). Using a number 8 punch, leaf disks were removed from the primary leaves, weighed and then floated on water at room temperature for a given number of hours in the dark. The experiment was terminated with the disks being

removed, patted dry with a filter paper and weighed. The disks were oven dried overnight and then weighed. The water content of the freshly sampled tissue was compared to its fully turgid state. The results were expressed on a percentage basis:

$$RT = [(FW-DW)/(TW-DW)] \times 100$$

where RT is equal to relative turbidity, FW is fresh weight, TW is turgid weight and DW is dry weight.

Results

The water saturation deficit in P. vulgaris primary leaves not exposed to air pollutants was first determined. The establishment of a water uptake pattern for 12 day old pinto beans was consistent with what Barrs and Weatherly (1962) showed for Ricinus communis, a rapid uptake observed in phase I due to a water deficit in the disks and a slow uptake in phase II due to the growth of the disks (Weatherly, 1950). Experiments were terminated at the end of phase I in order to determine the fully turgid value.

To measure the water deficit in plants under stress, water was withheld from 9 day old pinto beans. Samples were taken at 24 and 48 hours post watering. The results suggest that there was a slightly greater water deficit in moderately water-stressed plants than in unstressed plants (Table 1).

TABLE 1. Relative Turgidity of Well-Watered and Non-Watered Nine-Day-Old P. vulgaris Leaves

	Relative Turgidity	
	24 Hours Post Watering	48 Hours Post Watering
Control	76 \pm 1.63	88.2 \pm 2.36
Treated	73.4 \pm .64	85.3 \pm 1.39

The water status of plants exposed to HCl was also studied. Results indicate that among the 12 day old pinto beans exposed to high levels of HCl for 20 minutes, those exhibiting only slight damage had a smaller water deficit than control plants, at 0 hrs post fumigation. Twenty-four hours after the fumigation, there appeared to be little difference between control and treated plants. This suggests that plants initially respond to HCl gas by closing their stomates. (Table 2).

TABLE 2. 20-Minute HCl Fumigation at $19.8 \text{ mg HCl m}^{-3}$. Glazing and Less than 10% Necrosis Observed on Fumigated Plants

	Relative Turgidity	
	0 Hours Post Fumigating	24 Hours Post Fumigating
<u>Experiment #1</u>		
Control	82.2 ± 2.5	85.99 ± 2.09
Treated	85.13 ± 3.5	84.36 ± 8.6
<u>Experiment #2</u>		
Control	77.8 ± 2.4	76.5 ± 1.5
Treated	84.2 ± 6.7	82.1 ± 1.2
<u>Experiment #3</u>		
Control	84.66 ± 2.75	88.7 ± 2.59
Treated	90.28 ± 2.76	94.6 ± 4.58

Plants were also exposed to 15, 25 or 40 mg m^{-3} HCl for 20 minutes and then placed into groups according to visible damage (Table 3). The data shows that with increasing injury beyond 10% necrosis, there was an increase in the plant's water deficit when measured immediately after HCl exposure. Samples taken 24 hours after the fumigation exhibited relative turgidity values near control values suggesting recovery from the air pollutant stress.

TABLE 3. Relative Turgidity of 12-Day-Old *P. vulgaris* after 20-minute Exposure to Gaseous HCl as Related to Foliar Injury

Damage	Relative Turgidity	
	0 Hour	24 Hours
Control = 0	80.1 \pm 2	63.5 \pm 5
<1	83.3 \pm 3	61.72 \pm 3.4
1.5	74.6 \pm 3	65.9 \pm 1.6
2.5	77.9 \pm 3	55.6 \pm 1.4
3	68 \pm 13	65.8 \pm .62
4	75 \pm 1.3	66.9 \pm 1.25
(undamaged areas)		
4	56.3 \pm 21	
(damaged areas)		
5	41 \pm 25	

Free proline determinations were made first on control and on wilted plants. Water was withheld from pinto beans for 7 days to induce severe wilting. Proline levels were approximately 4 times higher in the wilted plants than the controls (significant at the 5% level). (Table 4 Experiment 1)

In the next experiments (Table 4, Experiments 2 and 3). 12 day old pinto beans were exposed to 26 mg m⁻³ of HCl for 30 minutes. Proline levels were determined at 0 and 24 hours post fumigation. Plants were scored for damage 24 hours post fumigation. HCl-treated plants that were visibly wilting at the end of the fumigation and exhibited moderate to severe damage 24 hours post fumigation had increased proline levels compared to controls at both 0 and 24 hours post fumigation. However, only the levels of the 24 hour samples were significantly (5%) higher. When treated plants showed no signs of wilting and only exhibited glazing 24 hours post fumigation, free proline levels were not significantly higher than controls, either at 0 or 24 hours post fumigation.

TABLE 4. Free Proline levels in Pinto Beans either exposed to wilting conditions or 26 mgm^{-3} HCl for 30 minutes.

Experiment	Treatment	Free Proline Levels (95% confidence interval for the difference between \bar{X}_{treated} \bar{X}_{control})		Damage
		0 hour	24 hour	
1	wilting	$1.47 \pm .63 *$		
2	HCl	$.094 \pm .099$	$.885 \pm .332 *$	wilting, glazing and necrosis
3	HCl	$.027 \pm .081$	$.037 \pm .067 *$	glazing only

In the next experiment, both watered and wilted pinto beans were fumigated with air, 16 mg m^{-3} and 43.4 mg m^{-3} HCl respectively for 30 minutes. Subsequent to the fumigation, half of the wilted plants from each treatment were watered. Free proline levels were determined at 0, 24 and 48 hours post fumigation. Results of experiment 4 are found in Table 5. At 0 hour post fumigation, there were no significant differences in proline levels among the watered and wilted control and HCl treated pinto beans. At 24 and 48 hours, free proline levels in the wilted set of plants (both controls and HCl treated) increased with free proline levels the highest in those plants exposed to 43.3 mg m^{-3} HCl.

In the last experiment (Figure 1), samples ranging from watered to water being withheld up to four days prior to treatment (in day intervals), were exposed to 16.8 and 43.4 mg m^{-3} HCl for 30 minutes. Both free proline and relative turgidity were determined for each sample at 0 and 48 hours post fumigation to 32 mg m^{-3} HCl for 30 minutes. For each sample class (plants minus water n days), the relative turgidity

TABLE 5. Free proline levels in watered and water-stressed pinto beans exposed to either 0, 16.8 or 43.4 mg m⁻³ HCl.

TREATMENT	MEAN FREE PROLINE LEVELS AT GIVEN TIMES AFTER HCl FUMIGATION (μ moles proline per gm fresh weight)							
	0 Hours				24 Hours			
	watered	wilted	watered	wilted then watered	watered	wilted	watered	wilted then watered
Air	.0264	.0449	.0614	.0297	.0579	.07648	.026	.445
16.8 mg m ⁻³ HCl	.0348	.0422	.0362	.0354	.0633	.0548	.068	.498
43.4 mg m ⁻³ HCl	.0443	.0626	.0504	.0275	.0996	.0396	.119	.787

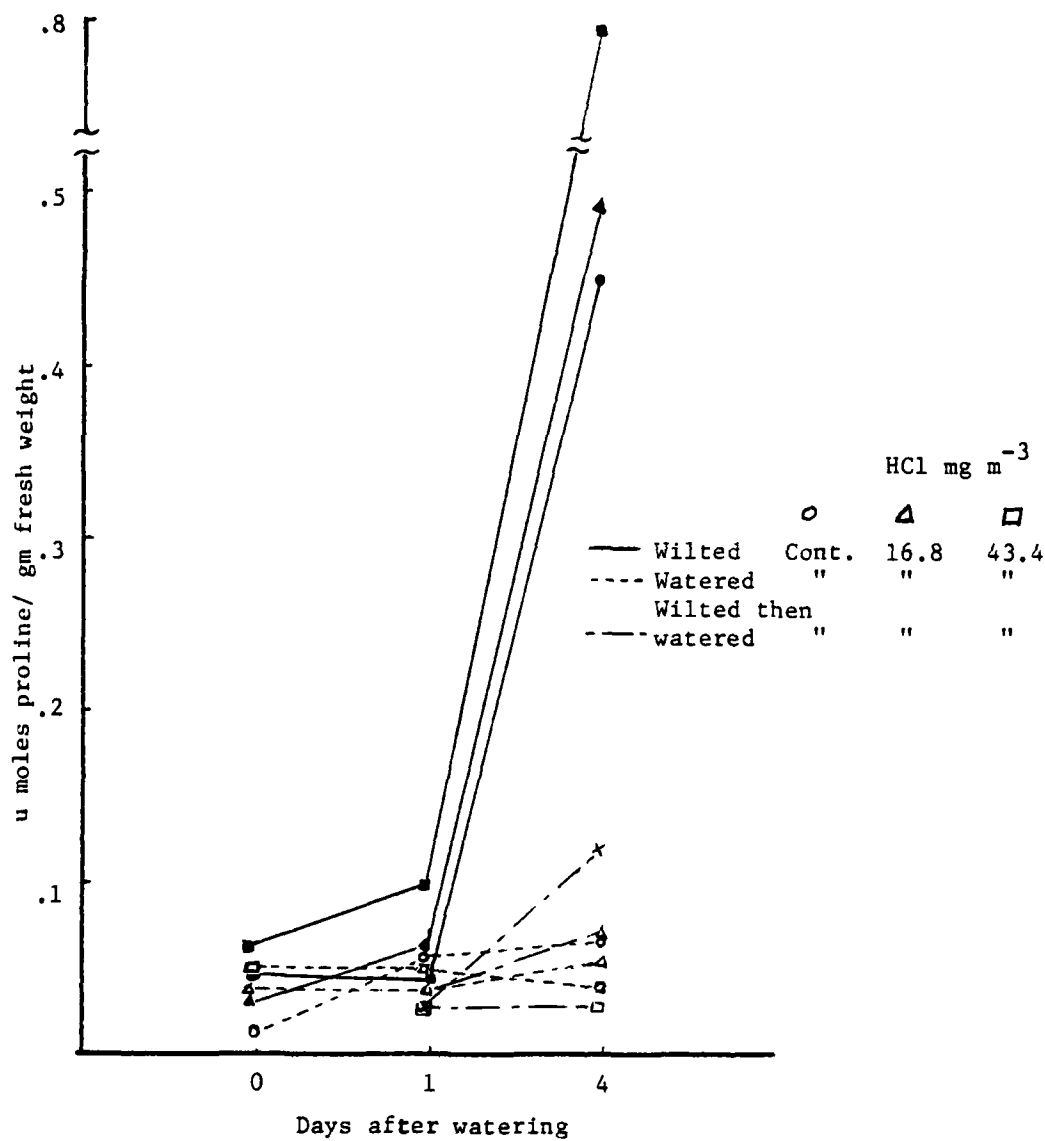


Figure 1. Response of proline concentration in pinto bean plants under different stages of water stress and exposed to two dosages of HCl.

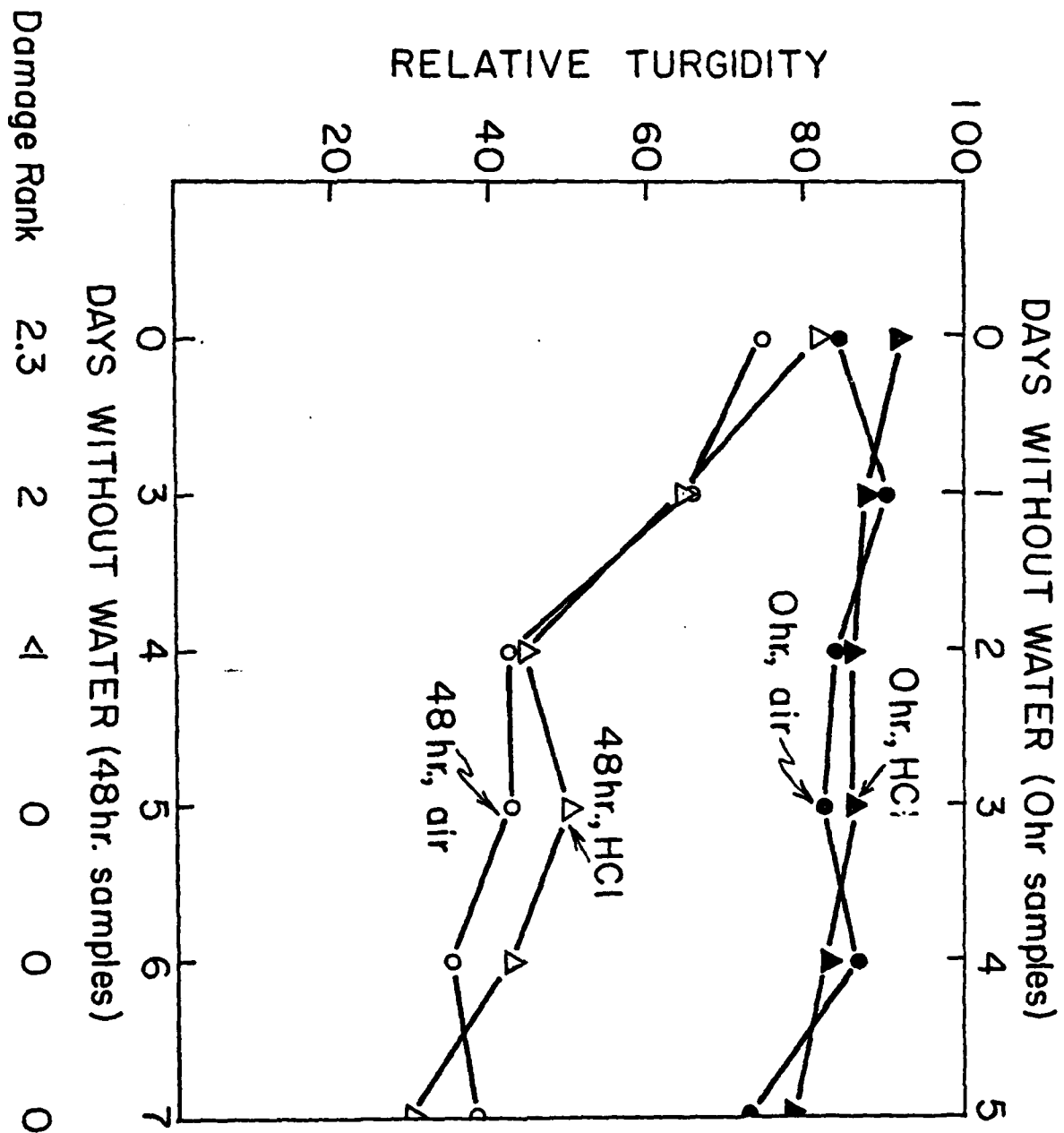


Figure 2. Changes in relative turgidity of pinto bean plants at 0 and 48 hours past fumigation with 32 mg m^{-3} HCl. Each sample class was 0 to 7 days after HCl was applied to the soil.

of treated samples was near or slightly less than that of controls at both sample times (Figure 2). At 0 hour post fumigation, free proline levels were significantly higher in HCl exposed plants than in controls (significant at the 5 and 1% level). At 48 hours post fumigation, there was a rise in proline levels in both control and treated plants, suggesting that water deficit rather than HCl was more important at this stage. In comparing the proline levels of pinto beans to their respective water status, free proline appears to be directly proportional to water deficit, with the highest levels being observed in the most water stressed plants (Figure 3). Also, plants with a water deficit of 50% or more appear to have little or no injury.

Discussion

Phaseolus vulgaris that had water withheld from them for one or two days, exhibited a greater water deficit than those that were watered daily. If well watered plants were exposed to gaseous HCl, plants that exhibited 10% necrosis or less had consistently smaller water deficits than controls had, those with greater than 10% necrosis had larger water deficits. This suggests that the plant's stomates are able to close in response to low levels of HCl thus preventing entry of the gas into the leaf and decreasing transpiration. However, if the concentration of HCl is sufficiently high, disruption of cellular membranes and metabolic activities may occur such that stomatal closure cannot occur. As a result, increased exposures to HCl are characterized by increased damage and water deficits.

Because the plant's water status is affected upon exposure to HCl, free proline levels were measured to determine whether the plant's response to

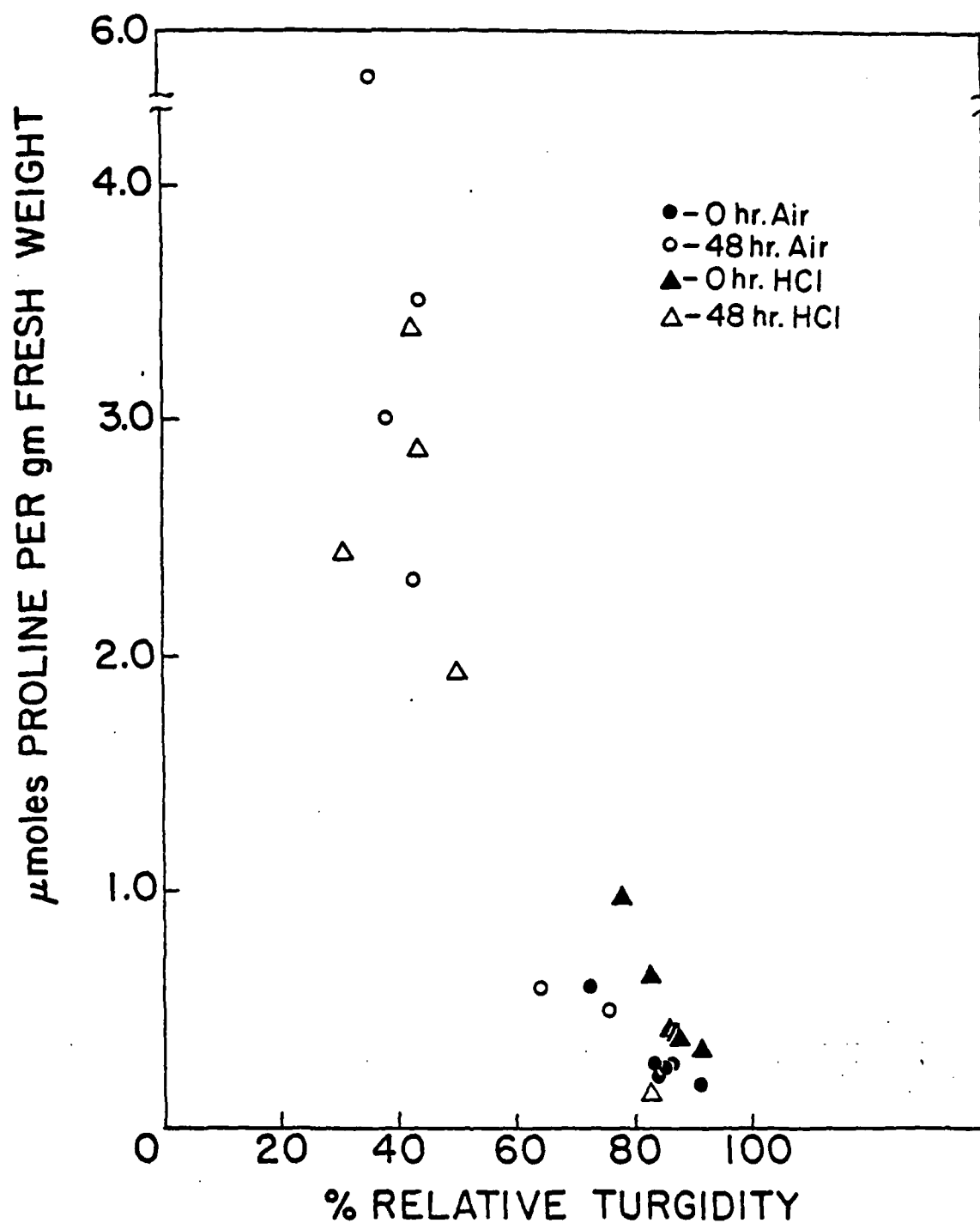


Figure 3. Changes in proline content of pinto bean plants in response to relative turgidity measured at 0 and 48 hours following treatment with air or with 32 mg m^{-3} HCl for 30 minutes.

HCl and water stress were similar. Prior to these experiments, free proline levels were observed to be higher in wilted plants than well watered plants, supporting findings that have been reported in the literature (Routley, 1966).

Proline levels among the fumigated plants were found to be the highest in those plants with the most visual injury and ultimately, the greatest water deficit. In experiments where both watered and wilted pinto beans were exposed to either 16.8 or 43.4 mg m⁻³ HCl, proline levels for each wilted group was higher than its respective watered pair. All the wilted groups (control + treated) and treated water groups had higher proline levels than the watered controls at 0 hours post fumigation. By 48 hours post fumigation, the highest proline levels were observed in wilted plants exposed to the highest levels (43.4 mg m⁻³) of HCl. The final set of experiments indicate that for Phaseolus, there is a direct relationship between free proline levels and water deficit. Although proline levels were significantly higher for all the HCl treated plants at 0 hours post fumigation, it is more than likely due to the water stress in the plant rather than a direct effect by HCl.

In conclusion, most of the experimental data collected suggests that HCl interacts with the plant's transpiration rates and that the free proline levels are a result of the plant's water status at any given time. A general consensus is that it is impracticable to use proline levels as a measure of the water deficit in plants because levels begin to rise when visual signs of water stress are apparent. Thus, free proline levels were not well suited to serve as a metabolic indicator of HCl damage.

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3. The relationship between water deficits and the injury response to HCl gas.

These studies were begun in the 1980 grant period and were continued through the summer extension. Eight and 12 day old pinto beans that were well watered then allowed to dry visibly wilt, and plants that were allowed to wilt then were watered were fumigated with 30 mg m^{-3} HCl gas for 45 minutes. At 24 hours post-fumigation, plants were scored for injury. Results are presented in Table 6. Experimental data indicate that both 12 day old pinto beans and agerium were more sensitive to HCl than 8 day old pinto beans; and, that for all plants, the treatment of wilting significantly reduced the amount of damage incurred from HCl exposure.

Phaseolus vulgaris were to be subjected to a water stress using polyethylene glycol and a semipermeable membrane system developed by Tingey and Stockwell (1977). The experiment was discontinued because weather conditions did not permit pinto beans to be grown in the required pine cells without wilting. As was shown in the first part of the experiment, wilting played a critical role in whether a plant would be damaged or not upon exposure to HCl gas.

Table 6. The influence of wilting on the injury response to HCl gas.

<u>Plant</u>	<u>Treatment</u>	<u>Average Damage Rank</u>	<u>Sample Size (# leaves)</u>
8 day Pinto Bean	watered	6.55	134
	wilted	5.11	108
12 day Pinto Bean	watered	10.86	108
	wilted	1.76	96
12 day Pinto Bean	watered	9.30	96
	wilted	3.03	110
12 day Pinto Bean	watered	10.9	40
	wilted, then watered	2.0	38
	wilted	1.83	36
Agerium	watered	8.21	24
	wilted	1.7	30

Leaves ranked using the Horsfall-Barrate scale:

1 = 0% damage; 2 = 0-3%; 3 = 3-6%; 4 = 6-12%; 5 = 12-25%; 6 = 25-50%; 7 = 50-75%; 8 = 75-87%; 9 = 87-94%; 10 = 94-97%; 11 = 97-100%; and 12 = 100% damage.

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Overall Review of the Grant (1976-1981)

THE IMPETUS

The total national annual cost of air pollution in the year 1968 was estimated to exceed \$16 billion (Barrett and Waddell, 1973). In subsequent years, environmental pollutants of considerable variety were increasingly cited as producing adverse effects on vegetation. The resultant potential for damage to economically important plants and agriculture is of major public concern. Gaseous HCl and similar oxidants are known to cause severe plant damage, but they are rarely considered as major pollutants because of their irregular and infrequent occurrence. The spector of significant plant damage is raised, however, by the increased use of chlorinated hydrocarbons and incineration of chloride polymers yielding chlorine and hydrogen chloride to the atmosphere. Further, some solid fuel rocket engines produce hydrogen chloride and hydrogen fluoride upon ignition. Some investigators have noted that hydrogen chloride is produced from particulate sea salt or particulate sodium chloride with either SO_3 or NO_2 (Erikson, 1959, 1960; Okita et al., 1974). If these observations are confirmed, coastal vegetation may be particularly susceptible to added burdens of HCl, especially if industrial, military, or municipal facilities, which generate additional HCl, are coastal in location. Consequently, hydrogen chloride may represent a major potential source of crop damage--a pollutant of future potential significance about which very little is presently known.

SPECIFIC AIMS

Specific questions addressed during the time period of this proposal are presented below:

1. Provide a description of histological, cytological, and fine structural characteristics exhibited by tissues exposed to hydrogen chloride.

a. What are the cytological and ultrastructural appearances of visibly damaged leaf tissues?

In this segment of the research program we characterized leaf damage induced by HCl with conventional light microscopic and electron microscopic techniques. We also compared our observations to those reported for other air pollutants.

b. Does the abaxial bronzing which is characteristic of hydrogen chloride damaged leaves result from cellular collapse and/or disruption? If so, which cells?

Hydrogen chloride injury is characterized by adaxial (upper surface) necrosis and bronzing at the abaxial (lower) surface of the leaf. Other air pollutants are associated with different symptoms, e.g., fluoride induces necrosis of leaf margins, SO₂ causes interveinal burns, ozone produces adaxial necrosis, collapse of some cells, and chlorosis of palisade parenchyma near stomata, and peroxyacetyl nitrate results in bronzing of the abaxial leaf surface (Middleton, 1961; Rich, 1964; Darley and Middleton, 1966; Heck, 1968; Granett and Taylor, 1976).

Abaxial leaf bronzing is characteristic of peroxyacetyl nitrate and hydrogen chloride damage. In the case of hydrogen chloride, abaxial bronzing may be the only visible damage symptom depending on plant age and concentration of the pollutant. One can envision various explanations

for the bronzing phenomenon. Epidermal cells could collapse allowing visualization into the intercellular air chambers of the spongy mesophyll. Alternatively, cells associated with intercellular air chambers immediately beneath stomata may collapse or be disrupted. This may or may not be associated with changes in the epidermis. A final possibility is that epidermal cells (or others) may accumulate some specific material. Bobrov (1952, 1955) indicated that abaxial bronzing on bluegrass and oats produced by peroxyacetyl nitrate resulted from collapse and disruption of cells associated with substomatal chambers. Whether HCl-induced bronzing occurs in a similar manner is unknown.

We examined HCl fumigated leaves to determine how the bronzing symptom is developed. Like previous workers we employed conventional microscopic techniques for such studies. In contrast to previous studies, however, we examined the developmental sequence and did not restrict ourselves to tissue in which bronzing is complete. Furthermore, we employed the electron microscope to obtain information regarding this point. To our knowledge, there are no previous ultrastructural examinations of bronzing in damaged leaves.

c. Are there ultrastructural changes in the absence of visible damage?

Our intent was to determine if fumigated leaf tissue lacking the visible damage symptomology exhibited hidden structural abnormalities. This study was primarily ultrastructural in scope. Plants were exposed to low levels of hydrogen chloride, levels insufficient to produce necrosis or abaxial bronzing, and were examined with the electron microscope. A comparison was made with nonfumigated plants to evaluate structural features.

d. What are the cell types most affected by hydrogen chloride?

Bobrov (1952, 1955) indicated that incipient damage produced by peroxy-acetyl nitrate occurred with collapse of cells lining the substomatal chambers. In a cytological study of soybeans exposed to ozone, Pell and Weissberger (1976) noted that paraveinal cells of the leaf were affected first and more extensively than palisade or spongy parenchyma cells. Because there are no detailed cytological ultrastructural studies of HCl-fumigated plant tissues, we could not anticipate whether a particular cell type is more sensitive to HCl than another. Such information is particularly important, however, in assessing how damage is produced by HCl. It must be remembered that our ultimate objective was to discover the mechanism of plant cell--HCl interaction. If a specific cell type is more sensitive than others, then clues regarding the mechanism of action can be obtained, especially if the cells involved have a specialized or peculiar function.

During examination of visibly damaged leaf tissue, we noted those cells which are more severely damaged than others to obtain an indication of cell sensitivity. In order to answer the question of cell sensitivity we employed several approaches, most of which were structural in orientation. In leaves exposed to relatively high HCl (25 mg/m^3) we examined tissue immediately after fumigation to determine if any particular cell type was severely damaged. This sampling was well in advance of development of visible symptoms. Consequently any collapsed, disrupted, or otherwise severely damaged cell may be more sensitive. Our second approach was similar except that the HCl concentration was low (approximately 8 mg/m^3). We expected that in this case, only the more sensitive cells would become damaged.

e. Does cell age influence sensitivity to the pollutant?

An early observation concerning oxidant damage to plant leaves was that it depended on age (Dugger and Ting, 1970a,b). Older, more mature leaves were injured more easily than young leaves by oxidant-type smog or ozone whereas peroxyacetyl nitrate affects only young leaves. Bobrov (1952), studying the effect of Los Angeles smog on oat leaves, observed that neither young nor mature leaves were smog susceptible and that only leaves which had just completed expansion were injured. The pattern of injury initially appeared as longitudinal bands corresponding to rows of stomata. With time the injury spread throughout the leaf. In a subsequent study with annual bluegrass, Bobrov (1955) observed that as leaves aged, smog-sensitive regions shifted from top to base. This distribution of cellular injury was accounted for by the fact that only recently matured cells showed injury. In young leaves, the tip cells have just reached maturity. In mature, recently expanded leaves, tip cells are senescent, mid-blade cells are just mature and smog sensitive, and basal cells are still meristematic. In old leaves, it is the basal cells that are just mature. From an analysis of age and smog damage in tobacco, Glaser et al. (1962) concluded that the most recently differentiated cells were most sensitive. MacDowall (1965) reported that tobacco leaves were sensitive to ozone just after leaf expansion. Cotton leaves have been reported to be maximally sensitive to ozone when about 75% expanded (Ting and Dugger, 1968). In a study by Davis and Wood (1973) with Virginia pine, it was observed that after 3-5 weeks of needle growth, cotyledon, primary, and secondary needle stages were injured by ozone. After 18 weeks, secondary, but not primary, needles became resistant. The younger needles were most severely injured at the tip in contrast to older

needles which were injured more at the base.

The correlation of age with sensitivity (amount of damage) was obtained from estimations of necrotic area on leaves fumigated at different ages. Similarly, reductions in the rate of leaf expansion or total leaf surface area were also related to age-influenced sensitivity.

- f. Does hydrochloric acid administration to leaves produce the same symptoms as hydrogen chloride?

Within the past several years numerous reports of rainwater with high acidity have been presented (e.g., Likens et al., 1972; Likens and Bormann, 1974). Values of pH as low as 2.1 have been measured and the acidity of the rainwater appears to exceed that expected for carbonic acid alone. Although the exact nature of the substances causing this excess acidity is not known with certainty, the generally accepted view is that it is caused by strong acids formed from industrial pollutants discharged into the atmosphere. Most of this acidity apparently results from sulfuric acid with less significant involvement of hydrofluoric and nitric acids. There is no evidence to indicate that hydrogen chloride cannot also be involved. Indeed, in instances of high humidity, hydrogen chloride would readily be converted to hydrochloric acid aerosol.

Deleterious effects of acidic precipitants on organisms have been recently reviewed (Likens et al., 1972; Almer et al., 1974; Likens and Bormann, 1974). Most of the experimental observations reported in the literature concern gross effects (Cohen and Ruston, 1925; Thomas et al., 1952; Sheridan and Rosenstreter, 1973; Kratky et al., 1974). Detailed morphological and anatomical studies have been reported for conifers (Gordon, 1972), yellow birch (Wood and Bormann, 1974), and pinto bean (Ferenbaugh,

1976) and only the latter publication reports information regarding physiological effects of acidic precipitants on flowering plants. To our knowledge, there are no reports dealing with hydrochloric acid mists.

In order to evaluate the similarities between hydrogen chloride- and hydrochloric acid-induced damage, we prepared hydrochloric acid solutions of variable pH and placed drops of these directly on leaves of test plants. The test plants were maintained in a semi-closed system to minimize evaporation with a consequent increase in acidity. Tissue subjected to acidic solutions in this manner was excised and examined with the light microscope following appropriate fixation and embedding procedures. This material was then compared with tissue fumigated with hydrogen chloride.

g. Which, if any symptoms are peculiar to hydrogen chloride?

Which seem to be pH effects?

Damage produced by hydrogen chloride may be largely attributable to its acidity. To test the relationship between pH and damage caused by hydrogen chloride, we applied other acids of variable pH to leaves and microscopically examined leaf structure.

h. Does cuticle thickness reduce leaf damage?

This was a light microscopic study of leaf tissues treated with drops of hydrochloric acid at varying pH. With this procedure we can determine if cuticles are damaged, what pH is necessary to damage the cuticle, and if cuticles reduce damage to underlying tissues. The plants for this experiment were selected for cuticle size. In addition, leaves can be treated to remove all or part of their cuticles. Leaves treated in this manner may also be used. We also considered using a scanning electron microscope to examine leaves for this investigation. With the scanning electron microscope, small.

localized areas of cuticle dissolution could be detected readily. Such would not be the case in sectioned, light microscopic material.

- i. Does the frequency of adaxial stomata correlate with leaf sensitivity?

Using drops of acid applied to leaves and usual microscopic techniques, we assessed whether greater damage occurs on leaves which contain more frequent stomata on the upper leaf surface.

- j. Do HCl fumigated leaves exhibit a wound response (e.g., corking around affected areas)?

We microscopically examined fumigated leaves to ascertain if one of the elicited responses is to form specialized thick walled cells around damaged areas.

Ozone damaged leaves of Citrus (Taylor, 1969) exhibit a wounding response characterized by "walling-off" of the damaged cells.

- k. What is the sequence of structural alterations in the development of damage symptoms?

Notably lacking in studies of phytotoxic responses to air pollutants are developmental considerations of the sequential alterations which produce observed symptoms. Most morphological and anatomical observations are restricted to tissues which are visibly damaged. Samples are usually obtained one or two days post-fumigation.

We felt that a sequential analysis of structural features taken from immediately post-fumigation until visible symptoms are expressed would be a significant contribution. Tissue samples taken immediately, 0.5 hour, 1 hour, 2 hours, 4 hours and 24 hours after hydrogen chloride fumigation provided considerable information along these lines.

2. What are the physiological characteristics of fumigated tissues?

a. Does HCl fumigation restrict leaf area? If so, by how much?

Several years ago in plots of Timothy grass treated with sulfuric acid solutions of pH 2.2-3.7, Cohen and Ruston (1925) noted a decrease in yield. Recent observations (Wood and Bormann, 1974; Ferenbaugh, 1976) have indicated that acidic precipitants, artificial or natural, produce deleterious effects on the developmental morphology of plants including stunted growth and interference with normal leaf development. There is currently no information on developmental abnormalities induced by hydrogen chloride other than that leaf and/or plant necrosis may result. There also is an important lack of information regarding growth of plants subjected to low levels of hydrogen chloride.

We initiated a study of total leaf area on fumigated pinto beans. The general protocol was to fumigate a large number of plants of identical age and measure daily for several days the leaf area on samples from this group. Experimental variables were leaf age and concentration of hydrogen chloride. With this approach we determined if leaf area of fumigated leaves differed from that of control leaves.

b. How is the rate of leaf expansion influenced by hydrogen chloride?

Answers to this question were obtained from the leaf area measurements just described. Since leaf area measurements are made daily, it is a simple matter to calculate the change in surface area per day and compare it with that from control plants. We were able then to distinguish between (a) reduced leaf expansion rate, but a longer growth period so that total leaf area was unaltered and (b) reduced rate of leaf expansion and reduced total leaf area.

- c. With respect to leaf expansion, what age leaf is most sensitive?

There are numerous indications that sensitivity to a variety of air pollutants is a function of plant age. Since we were primarily concerned with leaves, it was desirable that we determine at what age the leaves were most sensitive to hydrogen chloride. Plants in which leaf size or leaf expansion is most altered are those plants which exhibited greatest sensitivity at the time of fumigation. Furthermore, we routinely measured the area of necrotic lesions on leaves and presumed that the leaves with the most severe damage would be the most sensitive.

- d. Does hydrogen chloride reduce protein levels?

There appears to be some relationship between levels of some nitrogenous compounds and ozone injury. MacDowall (1965) reported that tobacco leaves were most ozone sensitive just after full leaf expansion and that sensitivity was associated with a decline in total protein. Ting and Mukerji (1971) suggested that free amino acids play a role in ozone sensitivity based on an observed decline in their concentration at about the same leaf age as that of maximum ozone sensitivity. While a considerable rise in free amino acids occurred 24 hours after fumigation (in cotton exposed to $1573 \mu\text{g}/\text{m}^3$ for 1 hour), Tingey et al. (1973) found a rise in amino acid concentration in soybean immediately after exposure which continued for at least 24 hours. With higher ozone concentrations, ($983 \mu\text{g}/\text{m}^3$ for 2 hours for soybean), the initial rise remained high for several days. Ting and Mukerji (1971) described similar results with high ozone concentrations for cotton. Craker and Starbuck (1972) also noted a rise in the free amino

acids in beans, but only after 24 hours using low ozone concentrations ($490 \mu\text{g}/\text{m}^3$ for 1 hour).

Ting and Mukerji (1971) reported a 17% decrease in total soluble protein of cotton leaves 24 hours after ozone exposure. The decrease in soluble protein was most dramatic in the chloroplast fraction. Craker and Starbuck (1972) also reported a decline in protein content in bean following exposure to ozone. Tingey et al. (1973), however noted that the level of soluble protein rose only after 24 hours following exposure at high ozone concentration. There was no change with longer ozone concentration.

Published analyses of proteins in tissues subjected to air pollutants are restricted to a consideration of ozone. No information is available for other pollutants. Ozone apparently affects protein metabolism either by enhancing protein hydrolysis, thereby producing an increase of free amino acids, or by interfering with protein synthesis without affecting amino acid synthesis. Declines in protein synthesis would follow disruption of the endoplasmic reticulum (the cellular organelle to which ribosomes are attached) or changes in levels of cytoplasmic ions necessary for ribosome and protein (enzyme) activity.

We examined leaf protein levels following exposure to high and low levels of hydrogen chloride. The basic format includes analysis of tissues for several days after HCl exposure. Consequently, we determined the immediate result of treatment on protein levels as well as subsequent changes. The latter were particularly significant with respect to recovery from treatments. The experimental techniques were straight forward and well established.

e. Does hydrogen chloride reduce the levels of ribonucleic acids?

Tomlinson and Rich (1968) and Pell and Brennan (1973) observed small declines in ATP levels in bean plants exposed to ozone. This decline was observed within 1 hour upon exposure and was interpreted as an initial response. Mudd et al. (1974) have shown that the nicotinamide ring of NADH is cleaved when ozone is bubbled through an aqueous system containing this compound. Since the ratios of NADH: NAD, NADPH:NADP, ATP:adenylates regulate all metabolism, it is likely that metabolism would be affected by nucleotide loss. The cell can make up for a net loss of all nucleotides by an increase in synthesis. Peroxyacetyl nitrate, however, merely oxidizes NADH (Mudd and Dugger, 1973). No information is available for other air pollutants.

Developmental studies concerned with changes in nucleotides during leaf development suggest that the period of ozone susceptibility corresponds to the time when nucleotide concentration is minimal. We attempted to determine how levels of nucleotides and ribonucleic acids were affected by hydrogen chloride treatment and whether nucleotide or ribonucleic acid level were correlated with sensitivity to the pollutant. Results indicated that the RNA assay was not sufficiently sensitive to answer our questions.

f. How are photosynthetic and respiratory activities affected by hydrogen chloride?

Important in vivo plant processes that can be inhibited by exposure to phytotoxic air pollutants are photosynthesis and respiration. Phytotoxic atmospheric pollutants have been rated by scientists as to their relative importance in affecting plants (Heck et al., 1973). Those recognized as

being of greatest significance are ozone, peroxyacetyl nitrates, nitrogen oxides, sulfur dioxide, and fluorides. Other atmospheric pollutants of lesser significance include chlorine, hydrogen chloride, and acid aerosols.

Ozone is rated as the single most important phytotoxic pollutant affecting vegetation (Heck et al., 1973). Respiration and photosynthesis were among the first responses to ozone examined (Todd, 1958; Todd and Probst, 1963; MacDowall, 1965b; Lee 1967; Hill and Littlefield, 1969; Pell and Brennan, 1973) and studies of photosynthesis and respiration still continue. The interaction of other air pollutants with plant activities, especially photosynthesis and respiration, is receiving considerable attention as well. At least six major phytotoxic air pollutants have been shown to reversibly inhibit apparent photosynthetic rates in plants (Hill and Littlefield, 1969; Hill and Bennett, 1970; Bennett and Hill, 1973). The relative amount of inhibition of photosynthesis after exposure to equal pollutant concentrations indicated the following ranking: $\text{HF} > \text{Cl}_2 \approx \text{O}_3 > \text{SO}_2 > \text{NO}_2 > \text{NO}$. Similar rankings of pollutant inhibition of respiration are apparently unavailable. The professional literature contains numerous publications dealing with inhibition of the photosynthetic and respiratory activities by air pollutants. We are aware of this extensive literature, but will not summarize it here because of its bulk. However, it is essential to recognize that only a small portion of the published literature is concerned with sub-toxic or cumulative considerations of sub-toxic exposures to plants, and even less is known about hydrogen chloride.

Considerable effort was devoted to examining hydrogen chloride interaction with photosynthesis and respiration. These studies examined oxygen consumption (respiration), oxygen evolution (photosynthesis), fixation of

carbon dioxide (photosynthesis), phosphorylation (respiration), photophosphorylation (photosynthesis), and other photosynthetic and respiratory features as a consequence of exposure to high and low hydrogen chloride concentrations. Furthermore, measurements were made on leaf discs obtained from fumigated plants, chloroplasts isolated from fumigated plants, and in vitro studies in which isolated chloroplasts and leaf discs from non-fumigated plants were exposed to hydrogen chloride.

g. Do stomates close during fumigation?

The role of stomates has been recognized since the beginning of air pollution studies with plants. Many research papers and review articles have been produced on the topic, most recently a detailed review by Mansfield (1973). We were concerned with the role of stomata in regulating hydrogen chloride uptake, whether stomatal activities impart resistance to the plants, what the effects of HCl on stomata are, and other considerations of gas exchange in leaves exposed to hydrogen chloride.

h. Are there net ion movements indicative of membrane damage?

Several studies on plants exposed to ozone indicate that ion and water movements indicative of membrane damage characterize ozonated plant tissues (reviewed by Heath, 1975). Our intent was to determine phenomena occurring as a consequence of hydrogen chloride exposure.

i. Are chlorides concentrated in leaves following fumigation?

What intercellular chloride levels are attained without development of damage symptoms? In what cellular compartment is the chloride localized?

High concentrations of hydrogen chloride provoke rapid, irreversible, and extensive leaf damage. In such circumstances, it is unlikely that leaves

accumulate measurable quantities of chloride. It was of interest to determine if chlorides are localized within a specific cellular compartment, and if visible injury resulted upon accumulation of some specific threshold quantity of chloride. With some fruit tree species this is about 2% chloride ion on a dry weight basis.

We examined these possibilities using 8-day old pinto bean plants. These plants were exposed to various levels of hydrogen chloride for 20 minutes ranging from 0 to 55 mg HCl/m³ in increments of approximately 5 mg/m³. After 24 hours, they were examined for bronzing and necrosis (i.e., visible damage), and leaf area was measured. Each treatment group was subsequently divided in half, with one subgroup washed to remove surface hydrogen chloride. The leaves were then homogenized and filtered through cheesecloth. The material retained by the cheesecloth, consisting largely of small leaf pieces and cells of the vascular system, was dried for one week at 70°C. The filtrate was centrifuged to obtain a pellet consisting of chloroplasts, nuclei, unbroken single cells, and cell wall fragments. This fraction was also dried. The resulting three fractions were then analyzed separately for chloride content.

This was a preliminary experiment to assess whether chlorides are accumulated. As a companion experiment, we examined the question of chloride localization using the electron microscope. Techniques are available to form electron dense precipitates of chloride with silver salts. With such a technique we were able with the electron microscope to visually determine where chlorides accumulate in fumigated tissues.

METHODS OF PROCEDURE

An important aspect in designing the program was that the most suitable plant species be used. We narrowed our consideration of various species to three: pinto bean (Phaseolus vulgaris), the American marigold (Tagetes erecta) and spinach (spinacia oleracea). The criteria employed for selection of these species were:

1. Availability of seeds
2. Rapid germination of seeds and growth of seedlings
3. That the species have been used in other investigations providing a foundation for the present work.

The quantity of histological, ultrastructural, and physiological studies employing pinto beans is too large to cite in this proposal. Particularly appropriate in this regard is that the primary leaves of pinto bean have been extensively employed in pollutant research. Marigolds and spinach have been less frequently examined in air pollution research. However, all three species have received some consideration in studies on hydrogen chloride toxicity (Lind and London, 1971; Lerman et al., 1975; Granett and Taylor, 1976).

We studied the ultrastructural feature of HCl-fumigated leaf tissues prior to the initial grant period. Our observations were largely directed to primary leaves of the pinto bean, but a preliminary survey of fumigated marigold leaves also occurred. Interestingly, the ultrastructure of fumigated marigold leaves differed from that exhibited by pinto beans.

In ultrastructural studies of pinto beans we noted considerable fine structural alterations as a consequence of HCl treatment and these have been observed much in advance of visual leaf damage. Palisade parenchyma cells in primary leaves of pinto bean sampled immediately after fumigation with HCl were crenulate and plasmolyzed. Cells of the spongy mesophyll

were also plasmolyzed. Membrane damage was prevalent in both cell types and the plasmalemma was often disrupted. Cytoplasm was characteristically vesiculate with vesicles of varied sizes and varied contents. Some vesicles contained a fibrillar material, others contained membranes and smaller vesicles, and some were apparently empty. Vesicular configurations of these types can be found throughout the cytoplasm of palisade and spongy parenchyma cells, but the multivesicular types were most prominent by the plasmalemma and tonoplast, both of which were frequently disrupted. Other organelles of the endomembrane system were altered as well. Endoplasmic reticulum and dictyosomes were rather uncommon and some profiles suggested that swelling of endoplasmic reticulum cisternae may provide the source for many of the observed vesicles. Mitochondria were conspicuous because of their highly irregular profiles and an apparent increased electron-transparency to the mitochondrial matrix.

Chloroplast ultrastructure was also sensitive to hydrogen chloride fumigation. Notably, chloroplast shape was convoluted and chloroplast envelopes were enlarged and vesiculated. In some cells, the outer membrane of the envelope seemed to be lacking. The most conspicuous feature of chloroplasts in fumigated leaf tissue was the presence of ordered fibrils throughout the stroma. These crystalline arrays were often massive causing distortions of chloroplast shape. Plastoglobuli, globular units of lipid, were frequently seen in normal chloroplasts, but in fumigated chloroplasts they appeared to be more abundant. Light capture and light-driven phosphorylation are functions of the membranes comprising the grana. In fumigated chloroplasts, the grana appeared unaffected except for those that were terminal. In those instances, the grana appeared swollen.

The cytoplasm of cells from fumigated plants and the organelles within these cells were also distinguished by the accumulation or association of an electron-dense amorphous osmiophilic material. Osmiophilic material was extracellularly deposited at the cytoplasm-cell wall interface and was observed adjacent to the exterior side of the plasmalemma in plasmolyzed cells. This amorphous osmiophilic material was also observed within the chloroplast envelope, within the mitochondrial envelope, within the nuclear envelope, and in association with the endoplasmic reticulum. In some perhaps exceptional instances, cisternae of the endoplasmic reticulum were observed to be totally occluded with this material.

No effects of HCl on epidermal or parenchyma cells associated with vascular tissues were discerned.

Tissue obtained from marigold leaves immediately after fumigation were ultrastructurally comparable to pinto beans as described above, with three notable exceptions: (1) crystalline inclusions in the chloroplast stroma were absent, (2) swelling was not limited to grana which were terminal, but occurred in virtually all grana, and (3) the swollen grana contained an electron-dense particulate.

Damage from HCl fumigation is usually assessed 24 hours after treatment. At this time bronzing, discoloration, and necrosis are evident. Ultrastructurally, leaf tissue sampled at this time was intermediate in appearance between tissue from control plants and tissue sampled at the end of the fumigation treatment. Most cells were no longer plasmolyzed. Central vacuoles of palisade and spongy parenchyma cells contained membrane fragments and a particulate, electron-dense material. Most membranes had apparently recovered from HCl stress as evidenced by the presence of

increased quantities of endoplasmic reticulum and dictyosomes and a reduction of the amorphous osmophilic material previously associated with the endomembranes. The most striking recovery of apparently normal structure was in the chloroplast. Chloroplasts no longer had crystalline aggregations in their stroma and the terminal grana were not swollen. Further, the vesiculation of the chloroplast envelope was reduced and their shape was generally normal. Many of the mitochondria remained crenulate with a relatively electron-transparent matrix. Other notable cytoplasmic features included a reduction of amorphous, osmiophilic material associated with membranes and the appearance of osmiophilic globules distributed randomly in the cytoplasm. A peculiar feature of this tissue was the prevalence of damaged cell walls. In numerous instances, cell walls were broken and in some cases, rather large segments were missing.

Ultrastructural alterations were noted in epidermal cells, particularly those at the abaxial side of the leaf. Some were collapsed and in many others the cytoplasm was considerably disrupted. In the latter, the cytoplasm was multivesiculate with electron-dense particulates within the vesicles. Additionally an electron-dense material was deposited along the inner surface of the cell wall as well as within the cell wall.

Spinach was chosen for the photosynthetic and respiration studies because of the following:

1. Spinach chloroplasts of high quality could be routinely isolated in large quantities.
2. The oxygen evolving abilities of spinach chloroplasts were fairly uniform, not only from day to day, but also week to week.
3. Spinach has long been the organism of choice for studies of

photosynthesis of isolated chloroplasts.

Use of the three plant species indicated above allowed us to address specific questions about the influence of HCl on the most appropriate tissue and by comparison identify those responses which are HCl-specific and which are species specific.

We examined 8-day old primary leaves of pinto bean exposed to 25 mg HCl/m³ for 20 minutes. At this exposure, 82% of all plants and 65% of all leaves were damaged, i.e., necrosis or bronzing or both. Since this exposure resulted in significant plant damage, we intended to continue our investigations utilizing this treatment level. However, for several of our intended experiments, this concentration was not suitable. In those investigations we anticipated 20-minute fumigations with approximately 8 mg HCl/m³ which is the maximum acceptable concentration of hydrogen chloride in the atmosphere according to the American Industrial Hygiene Association (Sax, 1963). We also anticipated using an intermediate concentration (approximately 12-15 mg/m³) for some experiments.

BRIEF SUMMARY OF EXPERIMENTS PERFORMED
DURING THE DURATION OF THE GRANT
(1976-1981)

MACROSCOPIC AND MICROSCOPIC RESPONSES

1. The impact of HCl gas on histological, cytological and fine structural characteristics of 8 day old pinto beans.

Foliar and microscopic observations of bean leaves exposed to hydrogen chloride, recovery from gaseous hydrogen chloride-induced stress in leaf tissue and on ultracytopathological characterization of leaves following short-term exposures to HCl gas are presented in the 1977 annual report.

2. Examination of the accumulation of chloride ions in primary leaves of pinto bean following fumigation with gaseous HCl.

Pinto bean plants, eight and 12 days from seeding, were exposed for 20 minutes to varying concentrations of HCl gas. At the end of the treatment, plants were returned to benches in the greenhouse. After 24 hours, these plants were examined for the presence of glazing on the lower leaf surface and/or necrotic lesions. The leaves were then harvested, frozen in liquid nitrogen, and ground while still frozen with a mortar and pestle. The frozen powder was placed in a beaker and solubilized with 10 ml. of deionized water. The macerate was stirred for 5-10 minutes and then allowed to steep for an additional 20-25 minutes. The homogenate was centrifuged, supernatant decanted, and the debris was extracted twice more with deionized water, 15 minutes each. Supernatants were obtained by centrifugation and were not combined so that extraction efficiency could be monitored.

The quantity of chloride ions present in the aqueous leaf extracts was measured with a Buchler-Cotlove Chloridometer (Model 4-2000). The quantity of extractable chloride expressed on a fresh weight basis increased in pinto bean leaves as a function of increasing HCl gas concentrations. This relationship was obtained with both 8 and 12 day old leaves, but the

quantities of chloride accumulated were consistently greater in the younger tissue. From this study it was apparent that the younger tissue accumulated more chloride, but that higher levels of accumulated chloride ions were necessary when comparisons of leaf injury were made. In other words, given equal levels of accumulated chloride, more of the 12-day old leaves were injured than the 8-day old leaves. This suggests that the twelve day plants are more sensitive of the two ages.

3. Relationship between leaf age and sensitivity to gaseous HCl.

Pinto bean plants were 6-12 days of age and a minimum of 60 plants of each age were treated. Following a 20 min. treatment with 25.6 ± 3.3 mg. HCl m^{-3} , plants were returned to greenhouse benches. Beginning 24 hrs. later, 10 plants from each age group were harvested daily for six consecutive days. The information recorded at each harvest for each leaf was (1) presence/absence of glazing, (2) presence/absence of necrotic lesions, (3) leaf area, (4) leaf fresh weight, and (5) leaf dry weight (after drying in an oven for three days at 70°C).

The primary leaves of pinto bean were increasingly sensitive to HCl gas within the limits of 6 and 12 days post-seeding.

Statistical analysis suggests that at this HCl concentration, injury to one-half of the leaves is probable when fumigated at 9-day age. Detailed results are described in Endress, Oshima and Taylor.

4. Sensitivity of Pinto Beans to HCl Exposure in Darkness.

Eight and 12-day old pinto beans were exposed to 19.2 mg HCl m^{-3} and 16-day old pinto beans to 18.3 mg HCl m^{-3} for 20 minutes in the dark. After fumigations were complete, greenhouse lights were turned on (1.7×10^4 erg/ cm^2 -sec intensity) until outside light reached the same intensity. All

plants were evaluated 24 hrs. after the fumigations. Data is reported in the 1978 Annual Report.

The percentage of leaves injured was only slightly reduced in dark fumigated, 12-day old pinto beans and was virtually the same as controls for 8- and 16-day old plants. However, the percentage of leaves exhibiting necrosis was reduced nearly 35% for 8 day plants and approximately 15% for 12- and 16-day old plants. Means of necrotic area/leaf were significantly less than controls at 5% level for 8-, 12-, and 16-day old plants fumigated in darkness. Among the age groups, mean necrotic area/leaf of 16-day old plants was significantly less ($p = .01$) than the 8- and 12-day old plants. These data suggest that stomatal closure may reduce HCl injury and higher HCl concentrations would be necessary when plants were in dark to obtain damage comparable to daytime fumigations.

5. Role of stomates in mediating severity of injury following exposure to HCl gas.

Pinto bean plants 8-, 12-, and 16- or 18-days from seeding were treated on their unifoliate leaves with various compounds to promote stomate closure. Leaf pairs were treated with Vaseline petroleum jelly, Absciscic acid (ABA), which is known to promote closure, at 3.78×10^{-5} M, or with Tween 20 (used as a surfactant). Leaf pairs were arranged in the following manner: 1) Control/Tween, 2) Control/ABA, 3) ABA/ABA, 4) Control/Vaseline (upper surface), 5) Vaseline (upper surface) Vaseline (lower surface), 6) Control/Vaseline (lower surface) and 7) Vaseline (both surfaces)/Vaseline (both surfaces). Tween was applied by dipping leaves in a solution of deionized water containing 0.01% Tween. Leaves were coated with Vaseline just prior to fumigations while abscisic acid in a solution of 0.01%

Tween 20 was applied either 0.5 or 3 hours before HCl exposures. In the initial experiment, 8-, 12-, and 16-day old plants were exposed to 18.3-19.3 mg HCl m⁻³. A second set of 12-day old plants was exposed to 36.1 mg HCl m⁻³ for 20 minutes and in a third trial, 12- and 16-day old plants were treated with 1.4 mg HCl m⁻³ for 120 minutes. In a further experiment, 8-, 12-, and 18-day old pinto beans were simultaneously exposed to 21.2 mg HCl m⁻³ for 20 min. ABA was applied 0.5 hr prior to HCl treatment. The simultaneous exposure of the three age groups was modified in a subsequent experiment by treating the leaves with ABA a minimum of three hours before HCl fumigation. In this experiment, 16-day old plants were used instead of 18-day old plants and the HCl concentration was slightly less (18.7 mg m⁻³). The presence of glazing on the lower leaf surface or appearance of necrotic lesions was determined 24 hours after fumigations for all experiments and ranked according to injury severity utilizing an arbitrary scale of 0 to 6:

<u>Rank</u>	<u>Injury Severity</u>
0	no visible injury
1	abaxial surface glazing only
2	>0-20% of leaf area necrotized
3	20-40% of leaf area necrotized
4	40-60% of leaf area necrotized
5	60-80% of leaf area necrotized
6	80-100% of leaf area necrotized

Results are described in the 1978 Annual Report.

Generally, treatments which prevented contact of HCl with leaves (Vaseline) or which closed stomates (ABA) reduced severity of injury but did not

necessarily decrease the incidence of injury. Age appeared to be an important interaction and can change relative leaf sensitivities to HCl gas. Development and function of stomata is known to be dependent on leaf age. Treatments intended to close stomata may have had no effect if stomata were immature or not developed. The injury response appeared to be mediated, in part, by the ability of gaseous HCl to diffuse through the stomatal opening. Observations from the dark fumigations support this hypothesis. However, solubilization of gaseous HCl in the leaf boundary layer may also be of importance in determining the degree of injury. The data suggest that HCl injury is a function of both stomatal opening and of physical contact but is, at best, highly variable and differs for each experiment and set of conditions, e.g., time of day, time of ABA application, HCl concentration and weather conditions.

6. Effect of Repeated HCl Exposures on Growth and Yield of Sweet Pea.

Sweet peas (Bijou dwarf Lathyrus odoratus var. Sapphire) were grown from seed and transplanted to 10 cm pots 14 days after seeding. Beginning at 6 weeks of age, half the seedlings were exposed to approximately 5 mg HCl m⁻³ once per week for 60 minutes, the others received filtered air. Harvests were performed once weekly and at least 24 hours after fumigations. Dry weights, leaf areas, and yield information was tabulated at harvest. The leaf-area ratio (LAR), relative growth rate (RGR) and net assimilation rate (NAR) were calculated for control and fumigated plants on a weekly basis and are reported in the 1978 Annual Report.

Results indicate that despite HCl induced foliar damage which, presumably, reduced photosynthetic capacity, the fumigated plants were

able to grow as effectively as non-fumigated plants. This is supported by the relative growth rate (RGR) data which is simply the increase in weight per unit of original weight over a time interval. The RGR curves for sweet pea were very close for both fumigated and control plants. This preliminary data suggests that growth of sweet pea was not severely hindered at the HCl concentrations which were used. It should be noted that this experiment was intended to run for a duration of 16 weeks. Fumigations were terminated prematurely because of problems with leaf necrosis and leaf drop from an unknown cause on non-fumigated control plants.

7. Differential Growth and Injury Responses of Pinto Bean Leaves to Gaseous HCl with Age.

Several experiments were performed to examine the role of leaf age in susceptibility to HCl toxicity and the effect a single gaseous HCl treatment has on subsequent leaf expansion. These studies have been analyzed and form the substance of a manuscript submitted to the Journal of Environmental Quality, 30 November 1977, entitled "Differential Growth and Injury Responses of Pinto Bean Leaves to Gaseous HCl with Age." A copy of this manuscript has previously been sent to the Air Force Office of Scientific Research.

8. Macroscopic and Microscopic Characterization of Aqueous HCl Injury on Pinto Bean Leaves.

Concentrations of aqueous HCl ranging from 1.03×10^{-3} to 1.03×10^{-1} M were applied to pinto bean unifoliate leaves in two ways. First, leaves were immersed in HCl (aq) containing 0.01% Tween in order to coat

the leaf with acid. Second, leaves were immersed for 20 min. in HCl with 0.01% Tween, removed, and excess HCl blotted off. Plants were sampled at 1, 4.5 and 24 hours after either treatment.

Macroscopic symptoms observed seemed to be more dependent on HCl concentration than on method of application. The same injury symptoms were observed for a given HCl concentration regardless of the application method although, in some cases, the 20 min. immersion gave a more severe expression of a specific symptom compared to the leaf dip. Both glazing and necrosis were noted in the HCl (aq.) treated leaves.

Microscopic observations were also made. Pinto bean unifoliate leaves (9 days old) were sampled by punching 7 mm diameter leaf discs and fixing them in 1) 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2 or 2) Karnovsky's fixative. After dehydration and embedding, sections were cut at 6-7 μ m with glass knives. These sections were stained with 0.5% Toluidine blue in 0.5% sodium borate alone or preceded by 1% Safranin in 95% ETOH (diluted 1:1 with distilled water) or stained in Jha's Methylene Blue. Microscopically, symptoms of aqueous HCl injury were very much similar to gaseous HCl. A detailed description of results is located in the 1978 Annual Report. This data seems to support the hypothesis that gaseous HCl is solubilized at the leaf boundary layer. Differences in intensity of injury between aqueous and gaseous HCl may be related to the degree of solubilization which is obtained at the liquid-gas interface of the boundary layer.

8a. Macroscopic and Microscopic Characterization of Acid and Saline Injury on Pinto Bean Leaves.

During the past grant period, the studies were extended to more fully understand the origin of the injury observed by treating bean leaves to

several different acidic and saline solutions. The objectives were to distinguish between injury due to pH or chloride ion and to determine if the injury was due to HCl specifically. For these investigations, the following acids and salts were used:

HCl	0.006 N	NaCl	0.01 M
	0.01 N		0.05 M
	0.06 N	LiCl	0.01 M
HNO ₃	0.01 N		0.05 M
	0.06 N	Distilled Water	
H ₂ SO ₄	0.01 N		
	0.06 N		

The unifoliate leaves from 12 day old pinto beans were immersed for 20 min. in each of these solutions. Excess liquid was blotted and the plants remained on greenhouse benches for 24 hrs. before injury determination. Leaf tissue from plants subjected to gaseous HCl for 20 min., as well as tissue from leaves treated with the acidic or saline solutions, were prepared for microscopic examination by fixation in glutaraldehyde followed by osmium tetroxide. Tissue sections were prepared by standard optical microscopic techniques as described in our previous reports and publications. Other leaf samples were prepared for examination by scanning electron microscopy to achieve greater definition of the surface injury.

The macroscopic appearance of the leaves treated with the acidic and saline solutions is summarized in the 1979 Annual Report. The symptoms expressed by leaves treated with the two saline solutions indicate that accumulation of chloride can be excluded as the cause of injury to the leaf surface. The various acids produced injury which was similar to each other and similar also to the injury present on leaves exposed to gaseous HCl.

9. Use of Vital Dyes to Detect Moribund Cells in Leaves Immediately After Treatment with Gaseous HCl.

As detailed in the 6 December 1977 proposal, we had expected to use vital dyes to identify cells destined to become necrotic from HCl exposure prior to development of visible injury. Light microscopic and ultra-structural examination of HCl-treated plants suggested that epidermal layers were particularly sensitive to gaseous HCl. Epidermal layers were removed from fumigated and non-fumigated leaves and incubated in solutions of neutral red, the vital dye selected. Stained cells (signifying they were living) were counted as a means of determining effects of HCl fumigation. We intended to examine leaf age, HCl concentration, and time following HCl treatment as variables. No significant differences were found, primarily because of the high variability in the number of vitally stained epidermal cells in control and HCl-fumigated epidermes. Consequently further studies with isolated epidermal layers were abandoned.

10. Fine Structural Assessment of Membrane Breakage Utilizing Lanthanum Nitrate

Electron microscopic examination of HCl-treated leaves demonstrated frequent membrane disruptions in tissue sampled immediately after treatment. Such disruptions were less frequently observed in tissue samples collected several hours after treatment. These observations suggest that injury to membranes may be partially reversible.

Frequency of membrane breakage and its repair can be examined by employing ultrastructural techniques. In a preliminary study, we examined membrane breakage and repair using lanthanum nitrate. Lanthanum molecules are of large size and consequently deflect electrons. Thus lanthanum ions

in tissue can be observed directly with electron microscopy. However, because of its molecular size, lanthanum cannot ordinarily pass the plasmalemma. It is therefore, usually restricted to the wall of plant cells. However, if breaks occur in the plasmalemma, lanthanum then penetrates into the cytoplasm. We incubated leaf discs obtained at various intervals following exposure to HCl gas in a solution of lanthanum nitrate. Appropriate controls were also employed, i.e., a) leaves not exposed to HCl gas, b) incubation solution lacking lanthanum, and c) incubation solution containing lanthanum and Triton X-100. Triton induces membrane disruption. The discs were then processed in the usual manner for electron microscopy and cellular distribution of lanthanum was observed.

The results of our preliminary observations are presented in the 1978 Annual Report, APPENDIX, Section B.

11. Reversible Alterations of Bean Chloroplasts Treated with HCl Gas.

Examination of ultrathin sections of HCl-treated pinto bean unifoliate clearly indicated that chloroplast ultrastructure was altered, most notably by the formation of stroma-localized crystals. However, as leaf tissue was sampled at progressively increased intervals after fumigations, it became obvious that these crystals disappeared. This phenomenon suggested that other reversible structural alterations may have occurred. Consequently, a more detailed analysis of such modifications was initiated. The study has been completed and a manuscript detailing the observations was submitted to the Botanical Gazette on 5 June 1978. A copy of this manuscript is in Section C of the APPENDIX, 1978 Annual Report.

12. Ultrastructural analysis of injury development in spinach
(Spinacea oleracea) and marigold (Tagetes erecta) exposed to gaseous HCl.

Marigold (Tagetes erecta L. cv. Senator Dirksen) and spinach (Spinacea oleracea L. cv. Bloomsdale) plants were grown in a greenhouse cooled by evaporative coolers equipped with activated charcoal filters. Marigold plants were 29 days post-seeding when treated with 27.3 mg. HCl m⁻³ (ppm HCl = 1.52 mg. m⁻³) for 20 min. and spinach plants were 121 days post-seeding when exposed to 30.4 mg. HCl m⁻³ for 20 min.

Immediately after exposure to HCl, plants were returned to greenhouse benches and samples of leaf tissue were taken from marigolds at 0.5, 5, and 18 hr. post-treatment and from spinach at 0.75, 2, 6, and 24 hr. post-treatment from the youngest, fully expanded leaves. The sample discs, 4 mm. in diameter, were cut from leaf laminas with a cork borer and immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 hrs. at room temperature. Following buffer rinses, the tissue was postfixed for 2 hrs. at room temperature in 1% osmium tetroxide in the same buffer, then dehydrated in an acetone series and embedded in epoxy resin (Spurr, 1969). Thin sections were mounted on bare grids and stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). A Philips EM-300 electron microscope operating at 60 kV was used to examine the sections.

Our observations of the spinach material were combined with those from the marigold tissues in a single manuscript which was published (Endress and Taylor, 1981). This manuscript describes our efforts to characterize the cellular injury, to compare the responses of these two species to each other and to pinto beans, and to compare the injury produced by supra-acute HCl exposures with previous reports of chronic HCl effects.

It was documented that cellular fine structure was altered more extensively than was anticipated from the visual injury symptoms present. The abnormalities displayed, particularly those associated with membrane fragmentation, cytoplasmic vesiculation and accumulation of electron-dense materials, were suggestive of repair processes occurring after exposure to HCl gas. Similarly, changes induced in the chloroplasts appeared to be transient. The increase in size and number of plastoglobuli and enlargement of granal compartments in marigold and spinach chloroplasts corroborates the earlier observations of Masuch et al. (1973) for chronic exposure of spinach and observations of Endress et al. (1979) for supra-acute exposure of bean to HCl.

13. Subcellular localization of peroxidase activity in leaves of *P. vulgaris* (pinto bean) and *Lycopersicon esculentum* (tomato) following treatments with ozone and gaseous HCl.

Peroxidase is an enzyme which is present in virtually all plants. Its precise function is unknown, but elevated peroxidase levels are known to be produced by environmental stress, e.g., cold, drought, hypoxia, salinity and air pollutants. We performed a major study to determine if peroxidase activity was elevated in bean and tomato leaf tissues devoid of macroscopic injury after exposure to either HCl gas or ozone and to assess the relative response of each species to these pollutants. This experiment involved several different treatments and is detailed in the following section of this report. Samples of leaf tissues were also prepared for ultrastructural detection of peroxidase. From such samples, we hoped to determine the cellular site(s) of peroxidase activity.

Peroxidase catalyzes the reduction of H_2O_2 to water by electron

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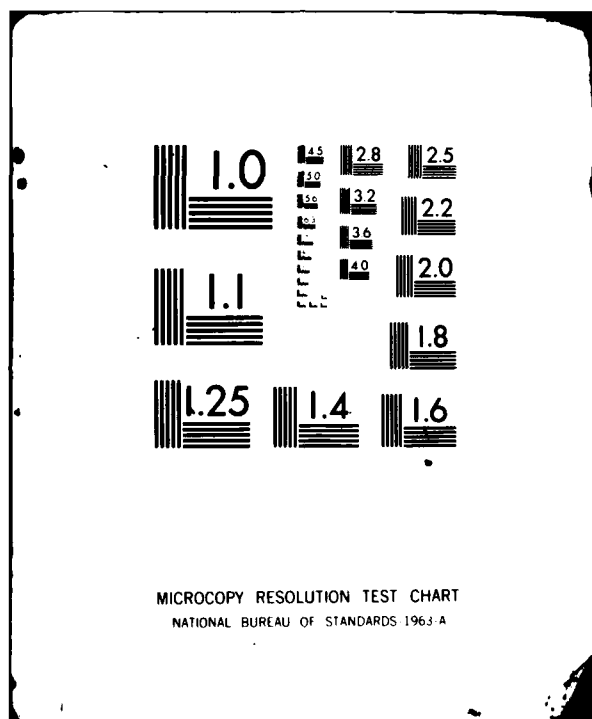
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donors other than peroxide. Localization of peroxidase at the ultra-structural level is not particularly difficult, but it is certainly tedious because of the necessity for numerous controls. Tissue from each treatment combination must be separated into 12 groups, each of which is prepared differently:

- (1) DAB (diaminobenzidine) + 0.002% H_2O_2 , pH 6.5
- (2) DAB only, pH 6.5
- (3) 0.002% H_2O_2 only, pH 6.5
- (4) DAB + 0.002% H_2O_2 + 0.1 M KCN, pH 6.5 (KCN inhibits peroxidase)
- (5) DAB + 0.002% H_2O_2 + 20 mM amino triazole, pH 6.5 (amino triazole inhibits catalase)
- (6) DAB + 0.002% H_2O_2 , pH 6.5 + boiled leaf tissue
- (7) DAB + 0.02% H_2O_2 , pH 10.0
- (8) DAB only, pH 10.0
- (9) DAB + 0.02% H_2O_2 , pH 10.0 + boiled leaf tissue
- (10) 0.02% H_2O_2 only, pH 10.0
- (11) DAB + 0.02% H_2O_2 + 0.1 M KCN, pH 10.0
- (12) DAB + 0.02% H_2O_2 + 20 mM amino trizole, pH 10.0

These are required in order to insure that the reaction product is specifically peroxidase. Catalase is also prevalent in plant cells and this enzyme catalyzes the reduction of H_2O_2 to water by either of two mechanisms:

(1) the peroxidatic reaction which requires an electron donor or (2) the catalatic reaction in which H_2O_2 serves as both hydrogen donor and acceptor. The two different pH concentrations and H_2O_2 concentration used above also served to distinguish sites of peroxidase activity from catalase activity -- slightly acid pH and low H_2O_2 concentration are optimal for peroxidase.

alkaline pH and low H_2O_2 concentration for catalase. Results are described in the 1979 Annual Report. No obvious differences were apparent which is not too surprising when the results of the peroxidase enzyme activity and isozyme study are considered. Those results indicated that peroxidase activity and isozyme pattern were quite sensitive to environmental stress, so sensitive that specific responses to gaseous HCl or ozone were masked by responses to handling a transpiration stress. Thus we questioned whether continued examination of the electron micrographs is warranted.

PHYSIOLOGICAL RESPONSES

1. Permeability Changes in Pinto Bean Leaves Exposed to Gaseous HCl

We have examined the permeability of pinto bean leaf cells exposed to gaseous HCl. In these experiments, 12-day old plants were treated for 20 min. with 22 mg HCl m^{-3} . Discs, taken from control and HCl-treated bean leaves immediately after, 24 hrs after, and 48 hrs after HCl treatment was terminated, were incubated in a solution containing ^{14}C -DOG. The amount of radioactivity incorporated was measured by scintillation counting. Results are published in Health and Endres, 1978.

2. Photosynthetic oxygen evolution by chloroplasts isolated from *P. vulgaris* and *S. oleracea* plants exposed to HCl gas.

Photosynthetic rates of 8-, 12-, 16-, and 20-day old pinto beans and of the three youngest fully expanded spinach leaves from the cultivars, "Melody", "Bloomsdale" and "Avon" were examined. Treatments for the pinto beans included the following: (1) 20 min. air chamber controls, (2) 20 min. fumigations with $21.79 \pm 3.5 \text{ mg HCl m}^{-3}$, (3) 20 min. fumigations with $33.09 \pm 6.8 \text{ mg HCl m}^{-3}$, (4) 1 hr air chamber controls and (5) 1 hr

fumigations with $7.25 \pm 1.8 \text{ mg HCl m}^{-3}$. *S. oleracea* plants had been exposed for 30 min. to either charcoal-filtered air or HCl gas (average concentration of 29.49 mg m^{-3}). In most experiments, two sampling times were used: immediately after and, 24 hrs after treatment.

The unifoliate leaves were removed from the plants, pooled into samples, deveined, weighed and homogenized using a small Waring blender. Samples were filtered through 4 layers of cheesecloth and then centrifuged. The chlorophyll concentration of each sample was determined as follows: 0.2 ml of sample was mixed in 4.8 ml of 100% acetone, centrifuged and the supernatant analyzed using the Beckman Acta Spectrophotometer at 645 and 663 nm wavelengths.

Following the determination of the chlorophyll concentration per sample, the rate of oxygen evolution ($\mu\text{M O}_2/\text{hr/mg chlorophyll}$) per sample was measured at 30°C using a Clark O_2 electrode connected to a Gilson oxygraph. Each sample of chloroplasts, obtained from one or two plants, was measured repeatedly four or five times to establish a mean rate of O_2 evolution/hr/mg chlorophyll for each sample. There were usually four samples per treatment. Results are published in the 1978, 1979 and 1980 Annual Reports and are in the manuscript, Endress, Suarez and Taylor, 1980.

The data collected not only confirms that exposure to HCl gas can reduce the ability of chloroplasts to evolve oxygen in both pinto beans and spinach but also indicates that differences between each cultivars exist.

3. pH dependent oxygen evolution of chloroplasts isolated from leaves of *P. vulgaris*.

The effect of pH on oxygen evolution from chloroplasts isolated from spinach was determined. These experiments were performed with the Gilson oxygraph so that simultaneous pH and pO_2 measurements could be taken. The control rate of oxygen evolution was determined and then acid was added to the reaction mixture. Both pH and pO_2 values were continuously recorded and the rates of oxygen evolution after addition of acid were expressed as percent of the control rate. Results are published in the manuscript Endress, Suarez and Taylor, 1980. The pH of the reaction solution markedly affected the ability of the chloroplasts to evolve oxygen. The phenomenon appeared to be a general pH effect as no differences were discerned between HCl, HNO_3 or H_2SO_4 acids. These results indicate that treatments which lower cytoplasmic pH in intact leaves could impair photosynthetic activity significantly.

4. Photosynthetic and respiratory rates in leaf discs from *P. vulgaris* plants exposed to gaseous HCl.

Experiments have been initiated to determine the consequences of HCl gas treatments on photosynthetic and respiratory rates in leaf discs from 12 day old pinto bean plants. Respiration and photosynthesis are measured manometrically with a Gilson differential respirometer and the results expressed as $\mu l O_2$ absorbed per hr per mg dry weight of tissue. Respiration rates reflect O_2 uptake in the dark and photosynthesis rates reflect O_2 evolution from tissue exposed to light. Results are published in the manuscript, Endress, Suarez and Taylor, 1980.

5. Photosynthetic rates in leaf discs from *P. vulgaris* plants dipped

in aqueous HCl.

Leaves were immersed for 20 minutes in one of the following solutions: distilled H_2O , 0.01 N HCl, 0.02 N HCl, 0.04 N HCl, 0.06 N HCl, or 0.006 N HCl. The leaves were then blotted dry. Photosynthetic measurements of discs taken 1 hr from the start of the experiment were performed. Results are in the manuscript, Endress, Suarez and Taylor, 1980.

6. The Effect of HCl gas on RubP Carboxylase Activity

Photosynthetic CO_2 fixation was followed by examining the activity of RubP Case from control and HCl-treated pinto bean plant leaves were homogenized and centrifuged; supernatants were analyzed by measuring the amount of $^{14}CO_2$ fixed from $NaH^{14}CO_3$. The specific activity of RubP Case was expressed as nanomoles enzyme/assay/mg protein. Results are published in Endress, Suarez and Taylor, 1980. In general, both 8 and 12 day old pinto beans sustaining injury had depressed levels of enzyme activity.

7. Injury susceptibility and sensitivity of radish and pinto bean plants withheld from water.

The role of dessication with respect to injury susceptibility and sensitivity was studied. A 6 x 3 factorial experiment (6 levels of water status, 3 levels of HCl concentration: 0, 7.97, 23.30 $mg\ m^{-3}$) employing 180 plants of a single species (10 plants/cell) was performed using P. vulgaris and Raphanus sativus (radish). All plants were 12 days since seeding when exposed for 20 min. to HCl gas and were watered daily with 1/4 strength Hoagland's nutrient solution. Beginning at 7 days of age, some plants were held daily from watering until exposure to HCl. After exposure plants were placed on benches in the greenhouse and one-half of the plants in each cell were watered. The presence of glazing and interveinal necrosis and the amount of necrotic leaf surface was determined

subsequently. Leaf areas were also measured. Data is summarized in the 1979 annual report.

For radish, the injury elicited (both glazing and necrosis) was correlated with gaseous HCl concentration. Other factors such as the interval since the last watering or whether plants were watered after HCl treatment were without effect. Beans differed, however, in that both desiccation level and post-fumigation watering influenced plant injury. The greater the interval between HCl exposure and last watering, the greater the resistance to injury. Similarly, plants watered right after exposure sustained less injury than those not watered.

8. Role of leaf cuticle in determining sensitivity to gaseous HCl in *P. vulgaris* and Citrus lemon.

Bean plants were selected on the basis of leaf uniformity and were either 8, 12, or 16 days of age (post-seeding) at exposure to gaseous HCl. Lemon plants were selected according to size constraints before HCl treatments and leaf plastochron indices were calculated. Leaf plastochron was rounded to the nearest whole number and only lemon leaves with a plastochron index of 8-10 or 23-25 were sampled for analyses.

After treatments, the plants were returned to greenhouse benches. From each treatment per experiment, 100 leaves were taken for extraction of surface wax and a minimum of 20 leaves remained on the plants to estimate injury severity. Foliar injury was measured 24 hours after treatment. Leaves were scored for the presence or absence of necrosis and/or abaxial glazing and the proportion of necrotic leaf surface area was estimated. Results are reported in Swiecke, Endress & Taylor in the 1980 Annual Report.

It is recognized that leaf anatomy/morphology has consequences on the photosynthetic activity of the leaf and that leaf morphology is varied depending on the impinging environment. These studies suggest that morphological structure influences plant responses to air pollutants. Environmental parameters may directly influence susceptibility by optimizing conditions for maximum impact and may indirectly influence susceptibility by altering leaf or plant morphology which in turn influences susceptibility.

METABOLIC RESPONSES

1. Changes in Chlorophyll, Protein, and Nucleic Acid Content of P. Vulgaris Unifoliate Leaves after Exposure to HCl.

Pinto beans, at 8-, 10-, 12-, 14- and 16-days of age, were exposed to gaseous HCl at a concentration of $21.83 \pm 5.6 \text{ mg}^{-3}$ for 20 minutes. Non-fumigated treatments included both air chamber controls and greenhouse bench controls. Each treatment consisted of 60 plants. Following fumigation, plants were sampled at 0, 2, 4, 7 and 24 hrs in order to monitor changes over a short period of time and to determine if there was any recovery due to HCl changes. For each of the three treatments at a given sample time, there were five groups, each with two plants. Two discs of a known area were removed from each leaf, one for chlorophyll analysis, the other for protein and nucleic acid analysis. Fresh weights of each group were recorded before analysis was begun. Results are reported in the 1978 annual report.

The possible effect of HCl gas treatments on chlorophyll levels was reinvestigated during the 1978-1979 grant period using leaf disks of larger sample size and fewer sampling times in an attempt to reduce some

of the variability obtained in the first set of experiments. The results of these experiments are summarized in 1979 annual report where chlorophyll content is expressed on the basis of leaf area, fresh weight, and dry weight. At best this data suggests a differential response, i.e., the effect of HCl exposure on chlorophyll content is dependent on the age of the tissue at exposure.

2. Peroxidase enzyme activity and isozyme pattern from *P. vulgaris* and *L. esculentum* leaves following treatment with ozone or gaseous HCl.

Plants of pinto bean and tomato were 12 and 88 days from sowing, respectively, when exposed to either ozone for 1 hr at 0, 196, or 392 $\mu\text{g m}^{-3}$ (1 pphm = 19.6 $\mu\text{g m}^{-3}$) or gaseous HCl for 20 min at 0, 4.08, or 12.52 mg m^{-3} (1 ppm = 1.52 mg m^{-3}). Immediately following exposure to the pollutants, the plants were randomly divided into three equal-sized groups. One group was sampled immediately for peroxidase activity. The remaining groups were returned to greenhouse benches. At 24 hr. post-fumigation, all plants were scored for injury and one group was sampled for peroxidase activity. The remaining plants were re-scored for injury at 48 hrs. post-fumigation and then sampled for peroxidase activity.

Unifoliate pinto bean leaves and the first fully expanded tomato leaves were deveined, individually weighed and homogenized. Total peroxidase (PRO) activity was estimated spectrophotometrically by measuring the change in absorbances at 430 nm in 1 min.

Isozyme patterns of treated and control PRO samples were examined with polyacrylamide slab gel electrophoresis (Davis, 1964). Samples were placed on a 4% stacking gel which overlaid the 9% separating gel. A current of 25 mamps was applied until the tracking dye, bromphenol blue,

ran off the gel. Immediately after electrophoresis, the gels were removed and incubated for 30 min. at room temperature in a freshly prepared reaction mixture that contained 0.03% 3,3-dimethoxybenzidine, 0.03% H_2O_2 , and 0.1 M Na acetate buffer (pH 4.5). After incubation, gels were transferred to 7% acetic acid for 1-3 min., washed thoroughly with distilled water, photographed, and dried. Results of this study are reported in Endress, Suarez and Taylor, 1980.

Clearly, elevated peroxidase activity results from several different types of environmental stress and in field situations, it would be extremely difficult to distinguish whether increased PRO activity resulted from lack of water, excess salt, exposure to an air pollutant, etc. in the absence of other specific symptoms. Furthermore, our experiments suggest that simple movement of plants and/or the transpirational water loss induced by the air flow in the exposure chambers elicited increased PRO activity which was comparable to that induced by the pollutants. Thus peroxidase activity appears to be extremely sensitive to the internal physiological condition of the plants.

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